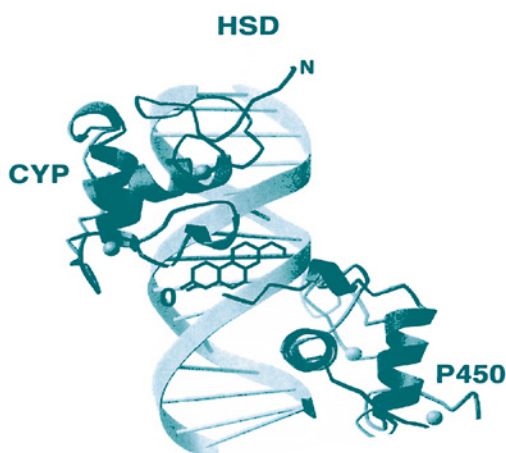


# GENETICS OF STEROID BIOSYNTHESIS AND FUNCTION

Intron 2  
(Exon 2)

518 bp CYP17 $\leftrightarrow$   
469 bp foreign DNA

-120



up478-479

n461Stop

del G438

5 Intron 7  
(Exon 7)

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# GENETICS OF STEROID BIOSYNTHESIS AND FUNCTION

Edited by

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# CONTENTS

Preface to the Series	vii
Preface	viii
List of Contributors	ix
1 Steroid Biosynthesis: Enzymology, Integration and Control <i>I.M.Bird and A.J.Conley</i>	1
2 Sterol Biosynthesis <i>D.Rozman and M.R.Waterman</i>	37
3 Vitamin D Biosynthesis and its Disorders <i>W.L.Miller and A.A.Portale</i>	57
4 Bile Acid Biosynthesis <i>J.Y.L.Chiang</i>	80
5 Cholesterol Metabolism in Steroidogenic Tissues <i>L.K.Christenson and J.F.Strauss, III</i>	114
6 Steroid 21-Hydroxylase <i>P.C.White</i>	145
7 Steroid 11 $\beta$ -Hydroxylase Isozymes <i>P.C.White and W.E.Rainey</i>	179
8 3 $\beta$ -Hydroxysteroid Dehydrogenase/ $\Delta^5$ - $\Delta^4$ -Isomerase Deficiency <i>J.Simard, M.L.Ricketts, A.M.Moisan and Y.Morel</i>	209
9 Human 17 $\alpha$ -Hydroxylase/17,20-Lyase <i>R.J.Auchus and W.L.Miller</i>	259
10 Aromatase: Insights into the Roles of Estrogens Revealed by Natural and Targeted Mutations <i>E.R.Simpson, K.Robertson, K.Britt, L.O'Donnell, M.Jones, A.Drummond, A.Thorburn and J.Findlay</i>	288
11 17 $\beta$ -Hydroxysteroid Dehydrogenase and 5 $\alpha$ -Reductase Deficiencies <i>J.Simard, A.M.Moisan, L.Calemard-Michel and Y.Morel</i>	298
12 Steroid Metabolism in Peripheral Tissues <i>C.L.McTernan and P.M.Stewart</i>	341

13	Animal Models of Impaired Steroidogenesis	362
	<i>K.M.Caron, T.Hasegawa, M.Bakke, N.A.Hanley and K.L.Parker</i>	
14	Regulation of Gene Expression by the Nuclear Receptor Family	373
	<i>S.Zoppi, M.Young and M.J.McPhaul</i>	
15	Neurosteroids and Brain Sterols	411
	<i>R.Lathe and J.R.Seckl</i>	
	Index	482

## PREFACE TO THE SERIES

The Modern Genetics series addresses new developments across the broad field of genetics. Recent volumes have been devoted to processes taking place at the level of the individual cell, exemplified by the pathways controlling programmed cell death (*Signalling Pathways in Apoptosis*, Volume 5), and at the whole organism level, represented by the genetic manipulation of livestock (*Animal Breeding: Technology for the 21st Century*, Volume 4).

The present volume covers an area of research that spans both levels of approach. Steroid signaling is widespread in nature; investigations cover the entire range—from physiological effects mediated by systemic hormones to the understanding of their modes of action at the molecular level. In medicine, steroids are among the most widely prescribed drugs for human use, while conserved steroid signaling pathways are present in vertebrates, plants, insects and fungi, presenting new avenues for biotechnological intervention.

The primary focus of this edition remains at the genetic and cellular levels, and with an eye to medicine and physiology, so emphasizing the well-established genetic systems of rodents and human. Unfortunately, perhaps, it has been impracticable to attempt to cover plants, insects and other organisms in one volume; their steroid pathways more than warrant coverage in a separate work.

J. Ian Mason, the editor of the present volume, has a long-established research reputation in the metabolism and action of steroids. He has taken on the formidable task of covering the major branches of steroid biology in mammals, and with particular attention to inherited gene lesions. To achieve this goal he has assembled an impressive panel of experts. The result is, we hope, an up-to-date and accessible overview of modern steroid biology that will be of interest and utility to experts and novices alike.

R. Lathe



# PREFACE

Steroids are crucial to a range of important physiological processes (glucose-insulin sensitivity, lipid and salt homeostasis, immune responses, reproduction/ pregnancy, digestion, bone growth, mood and memory function) and they are widely exploited as treatments for human disorders. Detailed understanding of the tissue-specific pathways of synthesis and degradation of steroids, and the closely related sterols, thus affords key insights into their physiological control and also illustrates novel possibilities for their therapeutic manipulation. In the last two decades, molecular biological technology has remarkably advanced knowledge of these processes at the level of the genome. It is now recognized that a steroid enzymic activity may be associated with a number of gene products, each of which may exhibit different levels of expression and regulation as well as manifestation at distinct tissue-sites.

The aim of this volume is to increase understanding of the structure, function and regulation of the enzymes, and the genes that encode them, that are involved in sterol/ steroid synthesis and metabolism in normal physiology, pathologies, and genetic disorders. The chapters comprise contributions from internationally renowned groups that we trust will be invaluable in providing deeper insight into the metabolism, disposition and function of sterols and steroid hormones. Wherever possible we have attempted to implement a common nomenclature of the steroid hydroxylases, dehydrogenases, receptors and their genes. Thus the GYP nomenclature has been adopted, in general, for the cytochrome P450 superfamily of enzymes, albeit that older nomenclatures have been preferred by contributors in certain instances for clarity purposes. In the case of the various steroid dehydrogenases, commonly used designations have been used notwithstanding that a number of these dehydrogenase gene products have been associated with various enzymic and functional activities.

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# 1. STEROID BIOSYNTHESIS: ENZYMOLOGY, INTEGRATION AND CONTROL

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*Biochemistry has a structure, a set of unifying themes, all the wonderful consequences of  
genetic control over the shape and function of proteins.*

*Albert L.Lehninger*

The purpose of this chapter is to introduce the process of steroidogenesis and describe the numerous aspects underlying physiologic control of steroidogenesis in different tissues and species. Rather than focus in detail on any one enzyme or aspect of steroidogenesis, our aim here is to emphasize that control of steroidogenesis can occur at many steps including enzyme levels, subcellular localization, cholesterol transport, relative substrate preferences, accessory proteins, control of electron supply, phosphorylation and selective inhibition by soluble gases. In particular, we assert that the concept of a single rate limiting step in steroidogenesis is hard to support and indeed any such rate limiting step so identified *in vivo* may quickly change with altered physiologic state. Finally, we attempt to place in context the lessons learned from knockout models, pregnancy and parturition. The goal is to leave the reader with a more complete understanding of how steroidogenesis as a whole is a finely controlled integrated process but at the same time is adaptable to a wide variety of different physiologic needs.

KEY WORDS: steroid biosynthesis, regulation, StAR, cytochrome P450, hydroxysteroid dehydrogenase.

## INTRODUCTION

Steroid hormone research began in earnest with the crystallization of sex steroid hormones from about 1929–1935, the glucocorticoids from 1935–1938, and finally of aldosterone in 1953. All possess the basic parent cyclopentanophenanthrene ring structure provided by cholesterol which is modified by an array of enzymes' expressed at varied levels in numerous tissues throughout the body. The remarkable features of steroid biochemical endocrinology include the profound change in bio-activity of the hormone products that

result from minor chemical modification of the basic ring structure, and the fact that this can take place locally in cells far removed from the site of substrate synthesis. Thus, physiological or pathological effects of steroids evident in organs and tissues are not necessarily reflected in, or predictable from, circulating steroid hormone concentrations. This additional regulatory plasticity, together with the lipophilic nature of the steroids themselves, has undoubtedly been a major factor in the adaptation of these hormones to the control of evolving processes in the radiation of vertebrate species. The generation and inter-conversion of steroid hormones is accomplished by enzymes that are encoded by an evolving group of genes (Baker, 1996; Nelson, 1999). Given the minute number of species investigated, there are many proteins, perhaps exhibiting unusual catalytic properties, that remain to be discovered.

The purpose of this chapter is to introduce the major enzymes characterized to date and to provide enough of their basic biochemistry, along with the anatomical distribution and biology of their expression, to allow an appreciation of how integration of function is ultimately achieved. Secondly, we will attempt to provide a balanced view of the contribution of genetics and molecular biology to our overall understanding of the many levels, and interwoven mechanisms, underlying control of steroid biosynthesis. There is no doubt that remarkable advances have been made using genetic and molecular approaches. But it is equally prudent to consider the interpretive limits of these approaches, and the models derived from them, in order to appreciate where the field might progress. For instance, gene knockouts can provide evidence corroborating the involvement of a protein in a process but may say little about how the process is regulated physiologically. Moreover, the extremes of steroid hormone synthesis and function seen even among mammals suggests that studies in any single species like the mouse may have little relevance to another such as human.

## THE ENZYMES

The enzymes involved in steroid metabolism can be divided into two broad groups, the cytochromes P450 and the oxidoreductases, each of which exhibits important, biochemically distinct properties. The cytochromes P450 (P450) were discovered and named based on a characteristic light absorption maximum at 450 nm when carbon monoxide is complexed with the reduced ferrous ion of the co-ordinated heme in the substrate binding pocket (Omura and Sato, 1964). Indeed, the ability of carbon monoxide, and other more physiologically relevant gases such as nitrous oxide, to bind and inactivate P450 (Estabrook *et al.*, 1963) and other heme-containing enzymes (cyclooxygenase, guanylate cyclase) may be of relevance in the regulation of enzyme activities *in vivo*. P450 enzymes comprise a large superfamily, the CYP superfamily (Nelson *et al.*, 1996), of highly conserved proteins (Graham and Peterson, 1999) that incorporate molecular oxygen into small lipophilic substrates with the provision of reducing equivalents from the cofactor nicotinamide adenine

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dinucleotide phosphate (NADPH). These reactions are essentially irreversible, not easily product inhibited and are so poised in the steroidogenic pathway that they determine the formation of each of the five major classes of steroid hormone: progestagens, mineralocorticoids, glucocorticoids, androgens and estrogens. Electron pairs must be transferred from molecules of NADPH to the P450 to activate oxygen in the substrate binding pocket, and this requires the participation of an additional one or two proteins acting as redox partners during the reaction. In effect, an enzyme complex forms transiently between the P450 and the redox partner (s) allowing for the effective transfer of electrons. There are two redox protein systems, one for the P450 enzymes anchored in the mitochondrial membrane, and another for all other P450s located in the endoplasmic reticulum (microsomal compartment). The mitochondrial redox support system (Lambeth *et al.*, 1982) consists of two components, a flavoprotein (FAD containing) adrenodoxin reductase, and an iron-sulfur protein adrenodoxin (ferredoxin). Microsomal P450s are supported by a single redox partner protein, the highly conserved flavoprotein (containing both FAD and FMN) NADPH-cytochrome P450 reductase (Porter, 1991). Thus, the sub-cellular location (Tamaoki, 1973; Lieberman *et al.*, 1984) and corresponding electron transfer or redox system defines a sub-classification of mitochondrial or microsomal cytochromes P450 involved in steroid synthesis, collectively known as the steroid hydroxylases.

Within the mitochondrial class of steroid hydroxylases of most species there are two, and in the case of human and rat, three functionally distinct P450 enzymes. The first, cholesterol side-chain cleavage P450 (CYP11A, also known as P450<sub>sc</sub>) utilizes cholesterol in the formation of pregnenolone, which is the universal precursor for all subsequent steroids. In other words, this is the first committed step and therefore CYP11A occupies a particularly important role in the regulation of flux through the steroidogenic pathways (Simpson, 1979). It is encoded by a single gene (CYP11A) as are almost all of the other steroid hydroxylases in the species studied to date (Miller, 1988; White, 1994). As expected, functional defects in the gene have dire physiological consequences. For instance, the anticipated effects on placental progesterone synthesis are assumed by some to be incompatible with the maintenance and survival of pregnancy in women (Miller, 1998). A second enzyme, cytochrome P450 11 $\beta$ -hydroxylase (CYP11B, also known as P450<sub>c11</sub>), catalyzes the last step in cortisol and corticosterone synthesis and it seems also to be encoded by a single functional gene (CYP11B1). In addition, CYP11B catalyzes the subsequent conversion of corticosterone to aldosterone in most species, and therefore this enzyme is critical in mineral metabolism. In humans and rats, however, a third mitochondrial cytochrome P450, aldosterone synthase (CYP11B2, also known as P450<sub>aldo</sub>), is encoded by another gene (CYP11B2) which has evolved by duplication of CYP11B1 to specifically catalyze aldosterone synthesis (see [Chapter 6](#)). While CYP11A is expressed in what are considered the classical steroidogenic tissues, i.e., the gonads, adrenal cortex and placenta of most species, CYP11B1 and CYP11B2 are expressed primarily in the adrenal cortex.

The enzymes comprising the microsomal steroid hydroxylase group include just three P450s involved in steroid synthetic steps subsequent to CYP11A that lead to both corticoid and sex steroid hormone synthesis. Progesterone and 17-hydroxyprogesterone are substrates for 21-hydroxylase cytochrome P450 (CYP21), which catalyzes the formation of



deoxycorticosterone and 11-deoxycortisol, intermediates in corticosterone and cortisol synthesis. As expected, the major site of expression of CYP21 is the adrenal cortex. Progesterone and pregnenolone are substrates for 17 $\alpha$ -hydroxylase/17,20-lyase cytochrome P450 (CYP17) in the synthesis of the C19 steroids androstenedione and dehydroepiandrosterone (DHEA), respectively. This enzyme exhibits two distinct activities as its name suggests. 17 $\alpha$ -hydroxylase forms hydroxylated intermediates for 17,20-lyase activity, subsequently catalyzing cleavage of the C17-C20 bond to form C19 steroids (Hall, 1991). The intermediate 17-hydroxyprogesterone is also released as a product in some tissues, and in the adrenal cortex of many vertebrate species it is the requisite substrate for CYP21 and thence cortisol synthesis. Thus, CYP17 is involved in both corticoid and sex steroid hormone formation. However, the adrenal cortex of other species, such as the rat and mouse, does not normally secrete cortisol and CYP17 expression, absent from the adrenal cortex, is almost exclusively restricted to the gonads and the placenta. Additionally, CYP17 shows an unusual degree of substrate specificity with respect to 17,20-lyase activity and thus C19 steroid synthesis, which is of considerable physiological and adaptive significance (Conley and Bird, 1997; Miller *et al.*, 1997). Although this will be discussed in greater detail later, at this point it can simply be noted that some species (human and ruminants) are relatively incapable of utilizing 17-hydroxyprogesterone for androstenedione synthesis. This contrasts with others (pig, rat, mouse, rabbit, horse) that can efficiently metabolize both 17-hydroxyprogesterone and 17-hydroxypregnenolone, and some, like the guinea pig, that do not efficiently utilize 17-hydroxypregnenolone for DHEA synthesis (Conley and Bird, 1997). In fact, the variation in ratios of 17 $\alpha$ -hydroxylase to 17,20-lyase activities sometimes encountered *in vivo* fueled debate over the possible existence and involvement of separate enzymes catalyzing each of these reactions (Hall, 1991). Both CYP21 and CYP 17 are encoded by single functional genes designated as CYP21 and CYP17, respectively. Finally, androstenedione and testosterone are utilized for estrone and estradiol (C18 steroid) synthesis by aromatase cytochrome P450 (CYP19, also known as P450arom; Simpson *et al.*, 1994). This enzyme is typically expressed at highest levels in the gonads and the placenta, but is also detected in numerous other tissues such as the brain, and peripheral sites such as adipose tissues, particularly in post-menopausal women. CYP19 is encoded by a single copy of the CYP19 gene in the mammals studied to date with the notable exception of the pig (Corbin *et al.*, 1995) which is believed to have multiple CYP19 genes encoding functional, tissue-specific isozymes (Conley *et al.*, 1997; Choi *et al.*, 1997; Conley and Walters, 1998). This is a most unusual occurrence which, like CYP11B1 and CYP11B2, may have significance in future studies into the evolution of steroid hydroxylase function (Corbin *et al.*, 1999).

The larger group of hydroxysteroid dehydrogenases or oxido-reductases is biochemically diverse (Baker, 1991) and genetically distinct from the cytochrome P450s. For instance, unlike the P450s which have evolved specialized steroid metabolizing functions, the oxidoreductases exhibit broader molecular binding specificities (Penning, 1999). While each of the P450s are encoded by genes that have for the most part been conserved in single copy, the oxidoreductases represent extensive evolution of function (Baker, 1996) by multiple gene re-duplication events. These enzymes catalyze dehydrogenation (reduction), hydroxylation (oxidation), and the isomerization and/or reduction of C=C bonds that

regulate the interconversion of active and inactive forms of steroids within each of the four major classes. In effect, these enzymes have the potential to influence the occupancy and subsequent activation of steroid receptors at any tissue site or cell in which they are expressed (Penning, 1997; Peltoketo *et al.*, 1999). Two major divisions can be recognized based on structural and functional grounds, the short-chain dehydrogenase/reductase (SDR) and the aldo-keto reductase (AKR) families.

The SDR and AKR families can be further broken down by functional properties into smaller groups. The main groups of enzymes within the SDR family include all but two of the known  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerases ( $3\beta$ -HSD; Abbaszade *et al.*, 1995), the  $11\beta$ -hydroxysteroid dehydrogenases ( $11\beta$ -HSD), most of the  $17\beta$ -hydroxysteroid dehydrogenases ( $17\beta$ -HSD) and the  $5\alpha$ -reductase enzymes. The aldo-keto reductase family includes  $3\alpha$ -hydroxysteroid dehydrogenases ( $3\alpha$ -HSD), the  $20\alpha$ -hydroxysteroid dehydrogenases ( $20\alpha$ -HSD) and at least one  $17\beta$ -hydroxysteroid dehydrogenase (mouse type 5) enzyme (Peltoketo *et al.*, 1999). Isozymes encoded by different genes have been identified within each group. Their number, tissue-specific distribution and catalytic properties vary with species to such a degree as to be well beyond the scope of this discussion. Subsequent chapters will provide details (see Chapters 8, 11 and 12). They are non-metallic enzymes that function as monomers or multimers but are not known to form functionally relevant complexes with other proteins, such as a redox partner. The fact that these enzymes have a different mechanism of action that does not involve a metalloprotein reaction center may also be relevant since it is known that NO, just like CO, can bind directly to and inhibit the reactive centers of cytochromes P450 and other heme-containing enzymes (Tsubaki *et al.*, 1987, 1990; Van Voorhis *et al.*, 1994; Del Punta *et al.*, 1996; Masuda *et al.*, 1997; Hanke *et al.*, 1998) but not, apparently, dehydrogenases. Further,  $3\beta$ -HSD, at least, has been shown to be inhibited by peroxide (Stocco *et al.*, 1993) but not NO. Thus the mixing of P450s and dehydrogenases in different steroid biosynthetic pathways provides opportunities for fine-tuning of end products through the production and presence of soluble gases and reactive oxygen species in steroidogenic cells.

The reactions catalyzed by dehydrogenases are generally reversible and therefore more easily influenced by co-factor abundance and product accumulation. Unlike P450 catalysis, both reduced and oxidized forms of nicotinamide adenine dinucleotide (NAD) and NADP participate as co-factors depending on the isozyme and oxidative or reductive nature of the reaction. Some are not even membrane associated.  $17\beta$ -HSD type I, that primarily catalyzes the conversion of estrone to estradiol, is cytosolic for example. Most others are anchored in the microsomal compartment of the cell, although there is evidence that  $3\beta$ -HSD may also be located in the mitochondria along with CYP11A (Sauer *et al.*, 1994; Cherradi *et al.*, 1994). Specific isozymes can be singled out as having particularly important functions: human  $3\beta$ -HSD type I catalyzes placental progesterone synthesis (Lorence *et al.*, 1990) and human  $3\beta$ -HSD type II catalyzes progesterone,  $17$ -hydroxyprogesterone or DHEA synthesis in the adrenals and gonads (Labrie *et al.*, 1992);  $11\beta$ -HSD type 2 metabolizes cortisol to cortisone in the distal nephron and collecting ducts of the kidney (Krozowski, 1999);  $17\beta$ -HSD types 1 and 3 synthesize estradiol and testosterone in the ovaries and testes (Anderson and Moghrabi, 1998; Peltoketo *et al.*, 1999); and  $5\alpha$ -reductase type 2 converts testosterone to the more active androgen dihydrotestosterone (DHT) in peripheral tissues (Russell *et al.*,

1994). While reviews of steroidogenesis seldom extensively cover members of the AKR family, studies into 3 $\alpha$ -HSD and 20 $\alpha$ -HSD suggest that these and other related enzymes offer great potential for modulating the effects of active steroids in a tissue-specific fashion (Penning, 1997, 1999).

### THE ROLE OF COFACTORS AND ACCESSORY PROTEINS

With relatively few exceptions (Hanukoglu, 1992), interest in steroidogenesis has focused on the cytochrome P450 enzymes or oxidoreductases themselves. In the case of the cytochromes P450, the essential redox transfer proteins are important, namely adrenodoxin and adrenodoxin reductase (supporting mitochondrial P450 activity; Lambeth *et al.*, 1982) and NADPH-cytochrome P450 reductase (supporting microsomal P450 activity; Shen and Casper, 1994). The possibility that proteins of the electron transfer system might limit P450 function, suggested earlier from studies in liver (Estabrook *et al.*, 1971), has been examined in a number of tissues: the human and bovine placenta, bovine and porcine gonads, and mouse testicular and adrenal tissues (Perkins and Payne, 1988), among others. The results of these experiments suggest that, unlike bovine placenta and ovary (Hanukoglu and Hanukoglu, 1986) or porcine ovarian tissues (Tuckey and Holland, 1989), the activity of CYP11A in human placenta is limited by the availability of adrenodoxin and adrenodoxin reductase in particular, increasing six-fold when these were added to saturation levels (Tuckey and Sadleir, 1999). The CYP19 activity in the human placenta and rat ovarian microsomes has been shown similarly to be stimulated by addition of reductase (Swinney *et al.*, 1993). The lack of sufficient redox partner binding proteins does not preclude product formation, but leads to an increase in the release of hydroxylated intermediates in the case of CYP19 (Sethumadhavan and Bellino, 1991; Swinney *et al.*, 1993). Because these additional products may have physiological or pathological effects of their own (the 19-hydroxyandrogens are hypertensive for instance (Sekihara, 1983)), further studies into the electron transfer proteins and redox system seem warranted. Furthermore, diseases associated with heterozygous null or partial function mutants of the redox partner proteins might be anticipated.

Typically, cytochromes P450 incorporate molecular oxygen into substrates by reactions that are absolutely dependent on efficient electron transfer from donor molecules (Ortiz de Montellano, 1989; Guengerich and Macdonald, 1990). As noted above, reductase performs this function in the case of microsomal P450s and adrenodoxin and adrenodoxin reductase does so for the mitochondrial P450s. In each case, these proteins must associate forming a transient complex with the P450 to express catalytic activity toward their substrates. In the absence of substrates, P450 can generate hydrogen peroxide and other oxygen radicals from reactions with water also bound in the substrate binding pocket. Otherwise, substrates enter the binding pocket of the P450 and the redox partner(s) associate, perhaps facilitated by electrostatic interactions, at a docking site on the exterior surface of the protein. This association allows the transfer of a pair of electrons from a donor co-factor, NADPH, the first of which goes to the ferric ion of the heme prosthetic group of the P450. Oxygen then binds the reduced ferrous iron and is activated during transfer of the second electron. Activated oxygen attacks the nearest carbon atom, hydrogen bonding and forming a hydroxyl group.

Hence one atom from the oxygen molecule is incorporated into the substrate (monooxygenase), the other is reduced to water; thus the use of the term mixed-function oxygenase applied to P450-mediated reactions. Such is the case for the formation of 11-deoxycortisol by CYP21, for instance (see [Chapter 7](#)).

In the case of other steroid hydroxylases such as CYP11A, CYP17 and CYP19, the formation of the final product involves an additional one or two hydroxylations performed in concert, after which the hydroxylated aliphatic side chain or group is cleaved (C27, C21 and C19 lyase or desmolase activities, respectively; Akhtar *et al.*, 1997). Thus, the general catalytic mechanism employed by these P450s is uniquely complex, because it necessitates the sequential transfer of 2 (CYP17) or 3 (CYP11A and CYP19) pairs of electrons during hydroxylation, consuming 2 or 3 moles each of oxygen and NADPH in the synthesis of 1 mole of final steroid product, pregnenolone (CYP11A), androgen (CYP17) or estrogen (CYP19). Debate still persists over the chemistry involved in the final hydroxylation and cleavage due to the lack of readily isolatable intermediates. However, it is clear that earlier intermediates are not only released, but that they have evolved a physiological purpose. Such is the case for 17-hydroxyprogesterone, released after a single hydroxylation cycle by CYP17, which has even been exploited to drive the synthesis of glucocorticoid (cortisol) in the adrenal cortex of many mammals (Conley and Bird, 1997). The fractional release of hydroxylated intermediates is very small in the case of CYP11A, but is higher in the case of CYP19, especially when reductase levels limit the rate of formation of active enzyme complexes with P450. In any event, the reactions catalyzed by the active P450 complex are equally dependent on the levels of reductase, substrate, oxygen and reducing equivalents.

The supply of pyridine nucleotide co-factors for support of steroid biosynthesis is an additional potential point of regulation that received a great deal of interest in the 1960s and early 1970s (Haynes, 1975). More generally, NADPH is generated in mitochondria and in the cytosol by distinct enzyme systems. In mitochondria, NADPH is replenished by the Krebs cycle components malate dehydrogenase (the malic enzyme) and NADP-linked succinate and isocitrate dehydrogenases. In addition, nicotinamide nucleotide transhydrogenase catalyzes the interconversion of NADPH and NADH, which is also generated by reactions within the Krebs cycle and fatty acid  $\beta$ -oxidation (Sazanov and Jackson, 1994; Hanukoglu and Rapaport, 1995). Whether or not the levels of mitochondrial NADPH limit steroidogenesis is unclear, but the rate of NADPH synthesis appears to be affected by steroid hormones (Lin *et al.*, 1974; El-Migdadi *et al.*, 1995). It is possible that changes in mitochondrial membrane potential or pH also influence reducing equivalent availability for steroid hormone biosynthesis. Since the mitochondrial membrane is impermeable to the movement of reducing equivalents, NADPH fueling microsomal P450 catalysis must be regenerated by cytosolic enzyme systems (Haynes, 1975). Glucose-6-phosphate (G-6-PD) and phosphogluconate dehydrogenase activities catalyzing glucose metabolism through the pentose phosphate pathway (PPP) are probably largely responsible, although cytosolic, NADP<sup>+</sup>-dependent malate and isocitrate dehydrogenases may also contribute to NADPH regeneration. G-6-PD, a soluble enzyme, is considered the rate limiting and regulated step in the PPP (Martini and Ursini, 1996) and the adrenal cortex is an abundant source of the enzyme (McKerns, 1965). Early studies demonstrated that ACTH stimulation of glucocorticoid synthesis was associated with a stimulation of G-6-PD activity

and G-6-P metabolism through the pathway (McKerns, 1965). The activity of the PPP in liver and other tissues is also influenced by nutritional state (Kletzien *et al.*, 1985, 1994), but similar data documenting the longer term effects of nutritional state on adrenal or gonadal G-6-PD activity or expression have apparently not been investigated. However, NADPH is one of only two high-energy phosphates that link anabolic and catabolic processes in the cell, and it would seem worthy of consideration and further study for this reason alone. Thus, it seems likely that the supply of reducing equivalents is linked with steroidogenesis as an integral component supporting substrate hydroxylation, but further studies are required to determine its role, if any, as a point of regulation.

#### CHOLESTEROL TRANSPORT VERSUS STEROID INTERCONVERSION AS THE RATE-LIMITING STEP

In addition to the identification and cloning of the P450s and dehydrogenases mediating steroid biosynthesis, early studies also demonstrated that pregnenolone and cortisol synthesis could be stimulated by aiding in the delivery of cholesterol substrates to the inner mitochondrial membrane. Subsequent studies over many years have culminated in the recent discovery of Steroidogenic Acute Regulatory Protein (StAR) by Clark *et al.* (1994). This protein is thought to be rapidly induced and mediates transport of cholesterol to the inner mitochondrial membrane by facilitating the formation of contact/transfer points in an area where CYP11A is also located. The process by which this occurs is clearly complex and still not fully understood but involves insertion to and transfer through the inner membrane with corresponding proteolysis and possibly phosphorylation (see particularly [Chapter 5](#)). The discovery of this protein answers many lingering questions and represents one of the most significant recent advances in steroid research. Unfortunately the results of studies performed in only a limited number of cell models, and particularly cell lines, have been extrapolated to suggest that StAR formation, and delivery of cholesterol to CYP11A, is the rate-limiting step in *all* Steroidogenic tissues (Orly and Stocco, 1999). There is abundant evidence however from the foregoing brief summary of the many enzymes potentially involved in steroid hormone biosynthesis and modification that there are many possible points at which regulation might be imposed. To determine the level at which regulation is truly exacted, it is first essential to properly define and understand the term “rate limiting”. The simplest definition of “rate limiting step” might be that it is the last point in a pathway at which substrate introduction fails to stimulate steroid synthesis downstream. Alternatively, it would be the only step in the pathway that, if changed even marginally, was accompanied by a correlated change in subsequent steroid output. In reality, these criteria have rarely been met for studies on steroidogenic systems *in vitro* or *in vivo*.

There are several additional reasons why CYP11A or even StAR cannot be the sole rate limiting step in the production of all steroid hormones all of the time. Firstly, a simple scheme involving regulation at a single point might adequately serve an unbranched synthetic pipeline leading to a single hormone product such as is seen for the monoamines. But steroid biosynthesis by pathways involving GYP17 in particular generates multiple products at different rates that are themselves changing and often not in the same direction. Therefore, by definition they cannot be controlled at one hypothetical rate-limiting step.

This becomes even more apparent when one considers the importance of ever-changing ratios of hormones controlling cellular endocrine inputs. If the rate of pregnenolone synthesis were the only regulated or rate limiting step, it would influence the production of all steroid products downstream equally and there would be limited opportunity for the modulation of steroid ratios and physiological response. There is a growing body of evidence to suggest that many if not all P450s may also be subject to phosphorylation control (Lobanov *et al.*, 1993; Miller *et al.*, 1997). Thus endocrine action via kinases may serve to alter flux through such branching biosynthetic pathways on a moment by moment basis and the specific kinases activated, and so P450s acted upon, will be agonist specific. This will also occur superimposed on the aforementioned possibility that relative flux through such branching pathways may also be subject to control by soluble gases as well as accumulating products acting to alter P450 versus dehydrogenase activities. Thus, a single rate limiting step means a single point of regulation and a restricted range of physiological adjustments.

Acute (within seconds) regulation of steroidogenesis does require mobilization of cholesterol to CYP11A and this is one reason why StAR is often cited as “rate limiting”. It is also noteworthy, however, that in many steroidogenic cell types initiation of an acute steroid response can occur some minutes before, and can be sustained for several minutes, without a detectable increase in StAR protein (Ariyoshi *et al.*, 1998). In addition, StAR inhibition or even StAR knockout (Caron *et al.*, 1997) is not always accompanied by a corresponding abolition of steroidogenesis in every organ. Thus while StAR or an equivalent protein is necessary for continued high level steroid production long term, its necessity for acute responses varies widely from cell type to cell type and even from agonist to agonist. Recent studies by Jefcoate and coworkers in particular have suggested that some of the discrepancies between levels of StAR message or protein and steroidogenic activity may be due to the fact that only a subpool of newly synthesised protein is actually involved in the cholesterol transfer process and the half life of the StAR protein subpool varies greatly from cell type to cell type (Artmenko *et al.*, 2001). Thus much of the confusion over the role of StAR in steroidogenesis may become clear in the near future. Nonetheless, in a multiple step pathway, such as that leading to corticoid synthesis in the adrenal zones, steroidogenic capacity of an *activated* pathway is most often limited by expression of a key enzyme, which may be CYP11A, CYP17, CYP11B1 or CYP11B2 depending on the zone and species. Many studies, particularly in sheep and cows, have further shown that it is CYP 17 which is rate limiting to cortisol biosynthesis and, more specifically, it is the CYP17:3 $\beta$ -HSD ratio in particular. CYP17 is very much regulated by pituitary ACTH while 3 $\beta$ -HSD is more constantly expressed. A fall in CYP17 expression (and so the CYP17:3 $\beta$ -HSD ratio) has been shown in many studies to be directly paralleled by a fall in cortisol and rise in corticosterone biosynthesis. (Conley and Bird, 1997). Similarly for ovarian estrogen biosynthesis, decreases in CYP17 and CYP19 are temporally associated with the decline in follicular estradiol production during the immediate pre-ovulatory period. No change is seen in CYP11A during this same period suggesting that it is not the regulated step in this instance (Conley *et al.*, 1994).

There are still further levels on which steroid biosynthesis can be controlled and integrated independently of StAR or even CYP11A. Only a few tissues are capable of substantial steroid synthesis *de novo* from cholesterol, while all others utilize substrates from

adjacent tissues, from the circulation or in the case of pregnancy, from another individual entirely. The rate of substrate supply to these tissues is sometimes critically affected by metabolism of potential substrates to other non-utilizable steroids. In other cases, the reverse is true, there is a necessity for deconjugation before some substrates can be utilized for instance and this may be limiting (Hobkirk, 1993). Thus, each cell, tissue and animal probably controls steroidogenesis at several steps. Moreover, different species are equally likely to have evolved alternative strategies for regulation to solve unique physiological problems. The major point to be made here is that statements indicating that specific steps, such as CYP11A or cholesterol transport to mitochondria often credited as rate-limiting, need to be carefully defined in terms of species, tissue, steroid hormone and physiological state. Additional committed steps exist in the synthesis of all classes of steroids, gluco- and mineralocorticoids, androgens and estrogens, and each is an equally probable candidate for control.

#### THE IMPORTANCE OF $K_m$ AND RELATIVE SUBSTRATE SPECIFICITY OF COMPETING ENZYMES IN DETERMINING THE END PRODUCTS OF STEROIDOGENESIS

The general properties of the enzymes catalyzing steroid metabolism are likely to have adapted to fill specific functional niches. Catalytic efficiencies and substrate specificity are broad but obvious examples. Steroid hydroxylases have evolved exhibiting higher affinities for a very limited number of substrates which are turned over at a relatively slower rate than are the P450 enzymes involved in detoxification of foreign chemicals in the liver. A change in apparent  $K_m$  or  $V_{max}$  of a steroidogenic enzyme would be expected to have an important impact on the ability of an enzyme to function in a specific cellular or tissue context. High substrate  $K_m$ 's (lower affinities) are typical of enzymes that are expressed early in the pathway and mostly in classic steroidogenic tissues that synthesize their own steroid substrates *de novo*. Essentially, the higher substrate concentration necessity for effective binding and catalysis by subsequent enzymes is suited much more to cells or tissues that synthesize their own substrate, the adrenal or gonadal tissues for instance. Enzymes later in the cascade, such as CYP19, and several of the oxidoreductase isozymes expressed in peripheral tissues, typically have very much lower apparent  $K_m$ 's, consistent with delivery of substrate synthesized in another organ or tissue. Dilution in the circulation dictates that substrates utilized for conversion in peripheral tissues will arrive in low concentrations. However, a low apparent  $K_m$  (high affinity) guarantees that enzymes can still be saturated, and efficient metabolism ensures a proper functional target tissue response. The estimated apparent  $K_m$  might be expected to reflect the substrate concentrations usually presented or available to the enzyme. Marked variation between the apparent  $K_m$  and the concentrations usually found in a tissue may in fact suggest that enzyme function is associated with utilization of a substrate perhaps not yet identified.  $V_{max}$  is less useful as an index of enzyme function, because it is more reflective of total enzyme levels, an index of the true metabolic capacity of a tissue.

Substrate specificity has emerged as a particularly important point at which regulation of steroidogenesis can take place. A prime example involves one particularly critical regulatory

juncture in the differential metabolism of pregnenolone (Conley and Bird, 1997). Theoretically, the utilization of pregnenolone by CYP17 first, and 3 $\beta$ -HSD subsequently, results in the synthesis of 17-hydroxypregnenolone, DHEA and finally androstenedione, referred to as the  $\Delta^5$  pathway. Otherwise, the initial metabolism of pregnenolone by 3 $\beta$ -HSD leads to progesterone which can be metabolized further by CYP17 to 17-hydroxyprogesterone and then to androstenedione via the so-called  $\Delta^4$  pathway. In other words, pregnenolone can be utilized by either CYP17 or 3 $\beta$ -HSD in reactions that compete for substrate, and what course pregnenolone metabolism takes can have major ramifications in terms of steroid output dependent on species (Fevold *et al.*, 1978). While no major functional differences between species have been reported for 3 $\beta$ -HSD, important species differences are evident at the level of CYP17 substrate specificity for pregnenolone or progesterone hydroxylation (Table 1.1), and particularly the subsequent 17,20-lyase step resulting in C19 steroid synthesis (Fevold *et al.*, 1989). CYP17 of all species appears fairly efficient at hydroxylating pregnenolone and progesterone. But this enzyme exhibits the ability to efficiently metabolize both 17-hydroxypregnenolone and 17-hydroxyprogesterone to DHEA and androstenedione, respectively, only in certain species (rat, pig, rabbit; Fevold *et al.*, 1989; Namiki *et al.*, 1988). In others, CYP17 is limited in the ability to perform the second hydroxylation and subsequent cleavage of the 17-hydroxyprogesterone intermediate. Specifically, human, cow, sheep and goat CYP17 can efficiently utilize 17-hydroxypregnenolone but not 17-hydroxyprogesterone (Zuber *et al.*, 1986; Lin *et al.*, 1991a; Barnes *et al.*, 1991; Imai *et al.*, 1993; Engelbrecht and Swart, 2000). In other words, androgen synthesis proceeds mostly effectively via the  $\Delta^5$  metabolic route. In contrast, the guinea pig exhibits a distinct preference for androgen synthesis through metabolism of the  $\Delta^4$  pregnanes, progesterone and 17-hydroxyprogesterone, but apparently not through the  $\Delta^5$  pathway (Tremblay *et al.*, 1994). This species-dependent biochemical characteristic of CYP17, favoring  $\Delta^5$  or  $\Delta^4$  metabolism for efficient androgen synthesis, makes the levels of 3 $\beta$ -HSD relative to CYP17 a critical determinant of the direction of steroid synthesis within the cell. Species exhibiting a predilection for  $\Delta^5$  androgen synthesis must constrain the expression of 3 $\beta$ -HSD in androgen secreting cells. Conversely, species with a bias toward  $\Delta^4$ -mediated androgen synthesis must ensure that there is adequate 3 $\beta$ -HSD expression for efficient androgen synthesis. Therefore the flow of steroids through either the  $\Delta^5$  or the  $\Delta^4$  pathway is dependent on the relative efficiency of the 17 $\alpha$ -hydroxylase and 17,20-lyase reactions, the competition between CYP17 and 3 $\beta$ -HSD for pregnenolone and the intermediates of  $\Delta^4$ / $\Delta^5$  metabolism. Thus, the characteristics of these enzymes, including substrate specificity of CYP17, catalytic efficiencies, as well as the relative levels or ratios of one enzyme to the other, determine the steroid flux through  $\Delta^5$  or  $\Delta^4$  pathways. An extreme example of this is seen in the Angora goat. As a result of extensive inbreeding, this animal exhibits a very poor stress response at the level of cortisol production and so has both low liver glycogen and higher use of amino acids for coat hair protein synthesis (Van Rensberg, 1970, 1973). The goat is another species that shows highly impaired  $\Delta^4$ -17,20-lyase activity and so cannot efficiently convert 17-hydroxyprogesterone to androstenedione. The low cortisol production in the Angora goat compared to the Boer goat or Merino sheep, however, is not due to poor ACTH secretion or deficiency of CYP11A, 3 $\beta$ -HSD or CYP11B expression/ activity. Instead this relates at least in part to a CYP17 with much stronger



Table 1.1 Bovine, ovine and porcine adrenocortical cell 17 $\alpha$ -hydroxylase activity against pregnenolone (P5) or progesterone (P4).

Treatment	Activity (nmol/mg/2h)					
	Bovine		Ovine		Porcine	
	P5	P4	P5	P4	P5	P4
Control	ND	ND	ND	2.119 $\pm$ 1.73	5.87 $\pm$ 0.91	ND
ACTH	36.38 $\pm$ 2.44	70.67 $\pm$ 3.5	12.31 $\pm$ 0.83	32.28 $\pm$ 2.43	33.13 $\pm$ 2.08	32.71 $\pm$ 4.69
All	0.95 $\pm$ 0.78	3.17 $\pm$ 1.89	ND	4.78 $\pm$ 0.70	11.32 $\pm$ 2.35	4.47 $\pm$ 3.65
ACTH/All	26.12 $\pm$ 0.59	60.86 $\pm$ 8.61	4.87 $\pm$ 0.22	7.61 $\pm$ 0.35	36.49 $\pm$ 1.1	33.24 $\pm$ 2.06

Notes

Adrenocortical cells were isolated from the zona fasciculata/reticularis by sectioning with a Stadie-Riggs microtome and trypsin digestion as described (Bird *et al.*, 1992). Cells were maintained in culture medium for 7 days before incubation in serum-free medium for 24 hours. Cells were then treated for 48 hours in serum free medium in the presence or absence of ACTH (10nM) and/or All (10nM). Medium was then removed and cells incubated either with 20 $\mu$ M pregnenolone with 2,00,000 dpm/ml [7-<sup>3</sup>H] pregnenolone (NEN-DuPont) in the presence of the 3 $\beta$ -HSD inhibitor 4 MA (Merck; 1 $\mu$ M), or alternatively with 20  $\mu$ M progesterone with 40,000 dpm/ml [<sup>14</sup>C]progesterone (NEN-DuPont) in the presence of the 11 $\beta$ -hydroxylase inhibitor, etomidate (Janssen; 1.5  $\mu$ M). After 2 hours incubation, medium was removed, steroid products extracted with dichloromethane, and extracts separated by thin layer chromatography as described (Bird *et al.*, 1992, 1995). Activity was calculated from the fractional conversion of substrate to 17 $\alpha$ -hydroxysteroid products in each case, as identified against authenticated standards. Results were normalized to cellular protein per well, and are the mean  $\pm$ SE of data from triplicate incubations. Data obtained in collaboration with W.E.Rainey and J.I.Mason.

substrate preference for pregnenolone together with relatively enhanced lyase activity (Engelbrecht and Swart, 2000). The greater ability of this CYP17 to strongly compete with 3 $\beta$ -HSD for pregnenolone substrate, combined with efficient lyase activity means far more DHEA is made at the expense of cortisol (Engelbrecht and Swart, 2000). Structural analysis of the goat CYP17 together with mechanistic studies to determine the ease with which the 17-hydroxypregnenolone intermediate is released from the enzyme would thus be of interest.

There are important consequences of the functional constraints imposed on androgen synthesis by the necessity for  $\Delta^5$  metabolism in humans, primates and ruminants in both the gonads and adrenal cortex. In particular, since 17-hydroxyprogesterone is not a viable substrate for androstenedione formation, its synthesis in the adrenal gland represents a commitment to glucocorticoid synthesis. In other words, the lack of  $\Delta^4$  lyase activity in the adrenal gland, together with a predominance of 3 $\beta$ -HSD activity over CYP17 promoting  $\Delta^4$  metabolism, provides a C21 steroid trap promoting glucocorticoid synthesis. This is facilitated further by the efficiency of utilization of 17-hydroxyprogesterone by CYP21. In species lacking a A block, the pig, horse and rabbit for instance, three factors might operate to promote efficient synthesis of cortisol: a high ratio of 3 $\beta$ -HSD to CYP17 (Conley *et al.*, 1994) which would encourage dominance of the  $\Delta^4$  pathway; a higher apparent Km for the lyase than hydroxylase activity of CYP17 (Hall, 1991) and slow cleavage of 17-

hydroxyprogesterone by allowing release from the substrate binding pocket; and lastly, the low apparent  $K_m$  of CYP21 for 17-hydroxyprogesterone, together with high levels of expression, would ensure that very little escapes metabolism for corticoid synthesis. The results of studies into CYP21 deficiency are consistent with the proposed role of CYP21 metabolism withdrawing steroid intermediates from the  $\Delta^4$  pathway. Consequently, the accumulation of 17-hydroxyprogesterone (accompanied by adrenal C19-steroid-induced virilization) in patients with congenital adrenal hyperplasia due to CYP21 or CYP11B deficiency (Tusie-Luna *et al.*, 1990; Strachan and White, 1991), more likely reflects the lack of catalytic activity of CYP17 toward 17-hydroxyprogesterone than it does any additional or particular defect in human CYP17 enzyme as previously suspected. Therefore, the ratio of the activities of CYP17 and  $3\beta$ -HSD in the adrenal glands can effectively promote or preclude efficient glucocorticoid or androgen synthesis in primates and ruminants since, especially with a  $\Delta^4$  block in 17-hydroxyprogesterone metabolism, the commitment is made at the point of pregnenolone utilization (Conley and Bird, 1997).

Several lines of evidence are consistent with the notion that the relative levels of CYP17 and  $3\beta$ -HSD play an important part in determining pregnenolone utilization, and so adrenal steroid secretion in humans, rabbits and ruminants. The need for high CYP17 expression to promote cortisol secretion in the face of high  $3\beta$ -HSD expression is supported by *in vivo* studies in the ACTH-stimulated adrenal gland of the rabbit which otherwise lacks CYP17 expression and secretes corticosterone (Kass *et al.*, 1954; Fevold, 1967). In addition, it is consistent with *in vitro* studies demonstrating that corticosterone secretion can be increasingly induced at the expense of cortisol in primary cultures of bovine and ovine zona fasciculata cells upon progressive inhibition of P450c17 expression (ovine, Rainey *et al.*, 1990; bovine, Rainey *et al.*, 1991). To this end, it is perhaps more than coincidental that  $17\alpha$ -hydroxylation can also involve either pregnenolone or progesterone to support cortisol secretion in most higher mammals, irrespective of  $\Delta^4$  17,20-lyase ability. Furthermore, human adrenocortical cells expressing a relatively lower  $3\beta$ -HSD:CYP17 ratio than bovine adrenocortical cells have in turn a correspondingly higher DHEA secretion rate (Hornsby and Aldern, 1984). Studies in human adrenocortical H295 cells have also shown that progressive attenuation of forskolin-induced CYP17 expression by co-treatment with angiotensin II (AII) results in a greater inhibitory effect on DHEA secretion than on cortisol secretion, thus demonstrating the lesser affinity and efficiency of the 17,20-lyase versus the  $17\alpha$ -hydroxylase reaction in the face of competing  $3\beta$ -HSD (Bird *et al.*, 1996a).

The balance between CYP17 and  $3\beta$ -HSD expression is equally critical for efficient androgen synthesis in male and female gonads of species with limited capacity for  $\Delta^4$ -17,20-lyase activity. An over-expression of  $3\beta$ -HSD would be expected to result in secretion of 17-hydroxyprogesterone in the absence of further metabolism toward glucocorticoid production. In other words, overexpression of  $3\beta$ -HSD in the gonadal tissues would be expected to lead to the synthesis and secretion of 17-hydroxyprogesterone. In fact, this appears to be the case for both men and women in which the circulating concentrations of 17-hydroxyprogesterone vary from 0.2–2 ng/ml, suggesting that the human gonad normally exhibits a relative over-expression of  $3\beta$ -HSD. As a terminal product of the human gonad, in the absence of adrenal dysfunction (Escobar-Morreale *et al.*, 1994), plasma concentrations of 17-hydroxyprogesterone reflect thecal or testicular rather than adrenal steroidogenic

function (Strott *et al.*, 1969; Smals *et al.*, 1980; Rosenfield *et al.*, 1994). In other words, the levels of 17-hydroxyprogesterone during the menstrual cycle represent diversion of steroid from the  $\Delta^5$  pathway, androgen and estrogen synthesis. Consequently, 17-hydroxyprogesterone concentrations are positively correlated with circulating estrogen levels during both the follicular and luteal phases (Strott *et al.*, 1969; Holmdahl and Johansson, 1972; Bosu *et al.*, 1972). Finally, if 17-hydroxyprogesterone secretion truly represents leakage from the  $\Delta^5$  pathway, that is, the over expression of  $3\beta$ -HSD activity in the ovary impairs efficient estrogen synthesis by reducing  $\Delta^5$  metabolism, limited inhibition of  $3\beta$ -HSD might even be expected to increase ovarian estrogen synthesis. Consistent with this proposal, treatment of macaques with trilostane, a  $3\beta$ -HSD inhibitor, during the luteal phase of the cycle resulted in a sustained increase in circulating estrogen levels, in addition to the expected reduction in progesterone (Duffy *et al.*, 1994). A similar result was obtained in cultured luteal cells (Duffy *et al.*, 1996). The lack of effect of trilostane on circulating corticoid concentrations in these animals suggests that  $3\beta$ -HSD levels in the primate adrenal gland are no more limiting in glucocorticoid production than they are in C19 or C18 synthesis. The lack of effect on glucocorticoid synthesis is perhaps because of the higher levels of  $3\beta$ -HSD expression in the adrenal zona fasciculata than the zona reticularis or the gonad. The similarity between men and women in the circulating levels of 17-hydroxyprogesterone (Anapliotou *et al.*, 1994) suggests a similar inefficiency in testicular androgen synthesis due to a relative excess of  $3\beta$ -HSD compared to CYP17 activity. Therefore, it might be anticipated that limited inhibition of  $3\beta$ -HSD in men could lead to an increase in testicular androgen synthesis.

The evolution of expression of CYP17 in the zona fasciculata and particularly the reticularis is notable because it introduces the potential for endocrine ambiguity. Specifically, those mammals exhibiting adrenal CYP17 expression are capable of significant extra-gonadal C19 steroid synthesis. Adrenal C19 steroid synthesis is not possible in many lower vertebrates, rodents and other species in which CYP17 expression is restricted to the gonads. As alluded to above, the epitome of this functional dichotomy is the human adrenal wherein the C19 steroid DHEA, and its sulphate, comprise the major steroids synthesized by the cortex (Labrie, 1991). Sulphation of DHEA, together with a relative deficiency of  $17\beta$ -hydroxysteroid dehydrogenase (necessary for testosterone synthesis) in the adrenal gland (Martel *et al.*, 1994), minimize the potential physiological impact of a prominent zona reticularis. In the absence of a substantial reticularis, circulating adrenal C19 steroids are low, of the order of 1ng/ml or less in plasma of pigs, sheep and cows (Baulieu *et al.*, 1967; Cutler *et al.*, 1978; Mostl *et al.*, 1981; Nathanielsz *et al.*, 1982; Stone and Seamark, 1985; Gaiani *et al.*, 1984; Belanger *et al.*, 1990). Therefore, although the expression of CYP17 is central to the regulation of steroid synthesis in either gland, it is also clear that mechanisms must operate to maintain a functional distinction between the adrenal cortex and gonad, without overly compromising steroidogenesis in either.

Over and above the species-specific substrate preferences of CYP17, the activity of this enzyme, and perhaps cytochromes P450 in general, can also be modified by other protein components of the microsomal environment in which it is expressed. This is particularly relevant with respect to 17,20-lyase activity of CYP17, but once again with apparent species differences (R.Estabrook, personal communication). NADPH cytochrome P450 reductase

and cytochrome  $b_5$  appear capable of stimulating 17,20-lyase activity and therefore increasing the ratio of lyase:hydroxylase exhibited by porcine CYP17 (R. Estabrook, personal communication; Hall, 1991; Katagiri *et al.*, 1982). Cytochrome  $b_5$  has a similar effect in the guinea pig adrenal microsomes (Shinzawa *et al.*, 1985; Kominami *et al.*, 1992) and supports androgen synthesis in rat testis (Ishi-Ohba *et al.*, 1984). A more profound effect was noted when human CYP17 activity was reconstituted with purified cytochrome  $b_5$ . Specifically, not only was  $\Delta^5$ -lyase activity promoted, but 17-hydroxyprogesterone utilization for androstenedione synthesis ( $\Delta^4$ -17,20-lyase) was demonstrated upon addition of cytochrome  $b_5$  to purified human CYP17 (Lee-Robichaud *et al.*, 1995). A role for cytochrome  $b_5$  as a mediator of human 17,20-lyase activity was further supported in studies employing immunoneutralization of the  $b_5$  protein in human adrenal adenomas (Sakai *et al.*, 1994) together with more recent discoveries of genetic defects among certain male pseudohermaphrodites. Specifically, some of these patients exhibit mutations in CYP17, but at sites on the protein thought to be involved in redox partner binding (Biason-Lauber *et al.*, 1997; Geller *et al.*, 1997, 1999). A single male pseudohermaphrodite has been identified with a mutation leading to a cytochrome  $b_5$  deficiency associated with a failure of virilization *in utero* (Giordano *et al.*, 1994). Interestingly, recent studies using recombinantly expressed proteins suggest that cytochrome  $b_5$  does not participate as an electron donor (Auchus *et al.*, 1998; Brock and Waterman, 1999) in contrast to conclusions drawn from earlier work in the pig CYP17 and the known effects of cytochrome  $b_5$  in other P450 enzyme systems (Vergeres and Waskell, 1995). It is important to note that while these data are consistent with an obligatory requirement for cytochrome  $b_5$  in 17,20-lyase activity and androgen synthesis in human tissues, the role of this particular factor as a physiological regulator awaits further study. Specifically, levels of cytochrome  $b_5$  would be expected to change along with 17,20-lyase activity in a physiological manner. In addition, reconstitution experiments using recombinant CYP17 and cytochrome  $b_5$  at various levels within a physiological range would go a long way to establish a primary regulatory role for this protein in the regulation of androgen synthesis in humans and other species. It is worth noting however, that cytochrome  $b_5$  did not alter 17,20-lyase activity in studies conducted on sheep adrenal CYP17 (Swart *et al.*, 1995). Therefore, the influence of reductase levels and/or cytochrome  $b_5$  on 17 $\alpha$ -hydroxylase: 17,20-lyase ratios of CYP17, appear likely to be highly species specific (R. Estabrook, personal communication).

#### CONTROL OF STEROIDOGENESIS BY TISSUE ZONATION

The functional implications of regulating steroid synthesis through differences in the relative levels of enzymes and associated proteins forms the basis of the anatomical, tissue-specific or zonal patterns of differentiation within the adrenal glands and the gonads, especially the ovary. In other words, a functional architecture based on the specific expression of key enzymes within particular tissues allows for compartmental regulation over steroidogenesis with a degree of independence that would otherwise be difficult to attain. At the organ level, this principally involves differences in the expression of various members of the cytochrome P450 families. The mature adrenal cortex is comprised of the zona glomerulosa, fasciculata and the reticularis, secreting mineralocorticoids, glucocorticoids

and C19 steroids, respectively (McNicol, 1992). The expression of CYP21 and CYP11B1, as well as CYP17 and CYP11B2 in certain species, is restricted to the adrenal cortex, these being the component enzymes of mineraloand glucocorticoid synthesis. In lower vertebrates, and some mammals such as rats, mice and rabbits, CYP17 and CYP19, as the major components of androgen and estrogen synthesis respectively, are expressed in greatest abundance in the gonads.

Zonation of the adrenal reflects the spatial distribution and expression of steroidogenic function (Sasano, 1994). While many steroid metabolizing enzymes are commonly expressed in both the zona glomerulosa and fasciculata (CYP11A, 3 $\beta$ -HSD, CYP21) the expression of CYP17 in higher mammals is more defined. Thus, the outer most zona glomerulosa, lacking CYP17 expression, is the major site of mineralocorticoids production, while the neighboring zona fasciculata, which expresses CYP17 in many higher mammals, synthesizes glucocorticoids. In domestic animals, cattle, sheep and pigs, CYP11B is expressed in both zones, catalyzing the synthesis of both aldosterone in the glomerulosa and cortisol in the fasciculata, but how these zone-specific activities are regulated is still something of a mystery. In other species, humans and rodents, the divergence of the CYP11B subserves zone-specific function. Expression of CYP11B2 and CYP11B1 is restricted to the glomerulosa and the fasciculata, respectively. Finally, the zona reticularis, not prominent in all species, expresses higher levels of CYP17 but no CYP21 and only low levels of 3 $\beta$ -HSD, and therefore produces a significant proportion of the C19 steroids secreted by the cortex of some species, particularly the human (post adrenarche) and non-human primates (Labrie, 1991). NADPH-P450 reductase is expressed throughout all zones of the adrenal cortex with the exception of a thin band of cells at the junction of the glomerulosa and fasciculata (Mapes *et al.*, 1999). Cytochrome b<sub>5</sub> expression is much more regional. Consistent with a function in support of 17,20-lyase activity, this protein is expressed almost exclusively in the zona reticularis of the primate adrenal gland (Mapes *et al.*, 1999). Other mammals known not to secrete adrenal C19 steroids have low or undetectable levels of cytochrome b<sub>5</sub> in the adrenal cortex (Conley, unpublished observations).

Studies of the endocrine control of adrenal zonation in the adult have been performed most thoroughly in human adrenocortical or H295R cells, as well as in bovine and ovine adrenal cells (previously reviewed in Conley and Bird, 1997; Bird *et al.*, 1998). In part, adrenal zonation relates to zonal changes in hormone receptor expression/agonist responsiveness. While ACTH receptors (activating the protein kinase A pathway) are expressed throughout the human/bovine/ovine zona glomerulosa (zg) and zona fasciculata (zf), there are clear zonal differences in AII Type-1 receptor levels (AT1-R, activating protein kinase C/Ca<sup>2+</sup>), as well as resting membrane potential (Mountjoy *et al.*, 1992; Bird *et al.*, 1996b; Quinn and Williams, 1992). Thus zg is most responsive to AII and K<sup>+</sup> (Ca<sup>2+</sup> signaling), while zf is less responsive to AII, with no K<sup>+</sup> response. While CYP11A, CYP17, 3 $\beta$ -HSD-II, CYP21, and CYP11B1 are all induced by increases in cAMP, studies with phorbol myristoyl acetate (PMA) alone or in combination with forskolin reveal subsets of steroidogenic enzymes regulated either positively (CYP21, 3 $\beta$ -HSD-II) or negatively (CYP17, CYP11A) by protein kinase C. Thus adrenal 3 $\beta$ -HSD-II and CYP21 expression is high in zg and zf, but CYP17 is not expressed in the zg where AII activation of protein kinase C is highest (Bird *et al.*, 1998).

In turn both  $K^+$  and AII-induced elevation of  $Ca^{2+}$  strongly induces CYP11B2 but not CYP11B1, consistent with preferential expression of P450aldo in the zg. This latter response appears to be mediated through CaM kinase. Therefore, differential signaling through kinase C and CaM kinases in addition to kinase A underlies, at least in part, zonal differences in both the early and late pathways involved in steroid hormone production within the adrenocortical zones of these species (Bird *et al.*, 1998).

The importance of AII acting through the AT1-R to attenuate/moderate CYP17 expression otherwise induced by ACTH is most clearly demonstrated in the sheep. While AII clearly stimulates a strong activation of phospholipase C and associated mobilization of  $Ca^{2+}$ , AII has no effect on acute cortisol production by ovine zfr cells (Viard *et al.*, 1990; Bird *et al.*, 1992). However, AT1-R is still expressed throughout the ovine adrenal cortex in an inverse level to 17 $\alpha$ -hydroxylase (Bird *et al.*, 1996b) and AT1-R still mediates the attenuation of ACTH-driven CYP17 expression in a similar manner to that seen in bovine and human cells (Bird *et al.*, 1992). Conversely, in the zona glomerulosa both AT1-R expression and  $K^+$  responsiveness are high and there is correspondingly no expression of CYP17 (Bird *et al.*, 1996b, 1998). Furthermore, species that do *not* express any CYP17 in the adrenal cortex and so make corticosterone as the major glucocorticoid also do not express, or express barely detectable levels of AT1-R in the zona fasciculata or reticularis (Bird *et al.*, 1990). An intriguing exception is the pig, which expresses CYP17 as well as AT1-R in the zona fasciculata/reticularis, and yet shows no evidence for AII-mediated attenuation of ACTH induced CYP17 expression (see Table 1.1), even though these same cells show increased expression of CYP17 in response to AII alone, as observed in bovine and ovine adrenocortical cells. Thus the pig does not show any need to constrain spill over of C19-steroid biosynthesis from the zona fasciculata and, in view of the ability to make C19-steroid via the  $\Delta^4$  and  $\Delta^5$  pathways, may even benefit from it. Perhaps this may be more than coincidence in view of the other unique feature of the pig such as multiple genes for CYP19 which could use these C19-steroid substrates and/or their metabolites (Conley *et al.*, 1996).

The importance of the relative levels of 3 $\beta$ -HSD and CYP17 in directing adrenal C19-steroid production is particularly evident during human development. The human fetal adrenal gland is unusually active in the synthesis of DHEA and its sulphate. Recent studies have determined that the fetal zone, which comprises the bulk of the cortex of the human fetal adrenal lacks significant expression of 3 $\beta$ -HSD (Doody *et al.*, 1990a; Mesiano *et al.*, 1993). The consequent lack of competition with CYP17-catalyzed  $\Delta^5$ -steroid metabolism results in unchallenged fetal adrenal DHEA production which remains unusually high as long as the fetal zone remains prominent. Higher levels of 3 $\beta$ -HSD are expressed as the definitive zone develops with increased cortisol and decreased DHEA synthesis (Mason *et al.*, 1993). In this regard, the low ratio of 3 $\beta$ -HSD:CYP17 in the fetal zone during human adrenal development, resembles the zona reticularis in adulthood. As noted above, recent studies suggest that cytochrome  $b_5$  expression further defines regions of functional differentiation within the non-human primate adrenal gland (Mapes *et al.*, 1999). Specifically, cytochrome  $b_5$  expression is restricted to the zona reticularis consistent with a functional requirement for 17,20-lyase activity and C19-steroid synthesis in the zona reticularis of the primate adrenal cortex. Unlike the human fetus, DHEA synthesis is relatively low during most of

fetal development in the rhesus macaque, but increases dramatically after day 160 of pregnancy (Siiteri and Seron-Ferre, 1981). This developmental increase in adrenal C19-steroid synthesis coincides with an increase in the expression of cytochrome  $b_5$  in the cells of the fetal zone, again consistent with development of androgen secretory ability as explained above (Conley and Mapes, unpublished observations). Therefore, the fetal zone of the rhesus macaque adrenal gland, though histologically prominent, is not as active in androgen synthesis as its human counterpart, and is not a functional homologue of the reticularis, until late in gestation when cytochrome  $b_5$  expression increases. Thus, the relative levels of expression of CYP17 and  $3\beta$ -HSD, in addition to the likely modulation of 17,20-lyase activity by accessory factors such as cytochrome  $b_5$ , are consistent with the principles of regulation of steroid synthesis described in preceding paragraphs.

Zonal expression of steroidogenic enzymes and proteins is equally obvious in the gonads, particularly the ovary. Estrogen production occurs in the wall of the follicle, which is compartmentalized both anatomically and functionally (Hillier *et al.*, 1994). Anatomically, the theca interna is separated from the stratum granulosum by a basement membrane. Functional differences between these tissues result from partitioning of receptors for LH and FSH, and each of these tissues responds to gonadotropin stimulation by the synthesis of different steroid products (Hillier *et al.*, 1994). Receptors for FSH are found exclusively on the granulosa cells and, in the early stages of follicular development, LH receptors are localized to the theca interna (Channing and Kammerman, 1974; Daguet *et al.*, 1979; Erickson *et al.*, 1985; Foxcroft and Hunter, 1985; Peng *et al.*, 1991). In all mammalian species so far characterized, androgen secretion, stimulated by LH, is essentially restricted to the theca interna (Erickson *et al.*, 1985; Meduri *et al.*, 1992, 1996). In contrast, granulosa cells are incapable of androgen synthesis, but under the influence of FSH they readily convert thecal androgens to estrogens (Hsueh *et al.*, 1984). The classic experiments by Falck (1959), transplanting either or both theca and granulosa cells together with vaginal epithelium to the eye of rats, demonstrated that efficient estrogen synthesis required synergism between these two cellular compartments. This concept, which was later termed the "two-cell theory" of follicular estrogen synthesis (Short, 1962), has been shown to be a general phenomenon characteristic of all mammalian species investigated to date (Armstrong *et al.*, 1981; Greenwald and Roy, 1994; Fortune, 1994). The "two-cell theory" is supported by the regional expression of steroidogenic enzymes. In most species, the theca interna is defined by CYP17 expression (Hanukoglu, 1992; Conley and Mason, 1993; Hinshelwood *et al.*, 1993; Richards, 1994), whereas the stratum granulosum expresses CYP19 (Masuda *et al.*, 1984; Rodgers *et al.*, 1986; Tamura *et al.*, 1992; Lautincik *et al.*, 1994; Conley *et al.*, 1994; Xu *et al.*, 1995). The pig presents something of an exception to this general picture. Results from several laboratories indicate that the porcine theca interna also possesses the ability to synthesize estrogens from androgens (Haney and Schomberg, 1981; Evans *et al.*, 1981; Hunter and Armstrong, 1987). These data have been confirmed at a molecular level showing that, although CYP17 expression is restricted to the theca as in other species (Conley *et al.*, 1994, 1995), CYP19 expression occurs in both theca and granulosa of the pig (Hunter *et al.*, 1994; Corbin *et al.*, 1995; Garrett and Guthrie, 1996, 1997).

The substrate preference of human, primate and ruminant CYP17 leading to the functional  $\Delta^4$  block to androstenedione synthesis has equally important implications for

steroidogenesis in the follicle, as it does in the adrenal cortex. In essence, the  $\Delta^5$ -pathway is not just adequate, but probably mandatory, for efficient androgen synthesis in humans, primates and ruminants that lack substantial  $\Delta^4$ -17,20-lyase activity under most physiological circumstances. At least two strategies can be envisioned to achieve a predominance of  $\Delta^5$  metabolism in the gonads of these species. Firstly, the relative expression of CYP17 compared to 3 $\beta$ -HSD might be predicted to be high in the gonads, in contrast to the adrenal cortex, to favor  $\Delta^5$  metabolism and efficient androgen synthesis. Indeed, the expression of 3 $\beta$ -HSD is low, and CYP17 high, during the follicular phase when estrogen synthesis is maximal in humans (Doody *et al.*, 1990b; Rheaume *et al.*, 1991), monkeys (Simard *et al.*, 1991; Martel *et al.*, 1994) and ruminants (Couet *et al.*, 1990; Rogers, 1990; Voss and Fortune, 1993a, b; Jeungel *et al.*, 1994). Alternatively, physical separation of CYP17 from 3 $\beta$ -HSD, and relegation of the latter enzyme to the site of aromatization would achieve the same end because it would ensure DHEA as the major C19-steroid synthesized in the theca. Thus, limiting the expression of CYP17 to the theca interna and 3 $\beta$ -HSD to the granulosa layer, may represent an additional strategy adopted in the bovine follicle to bring about efficient preovulatory estrogen synthesis (Conley *et al.*, 1995). Clearly, while a useful general model for ovarian steroidogenesis, the two-cell hypothesis based on the delivery of  $\Delta^4$  substrates from the theca to the granulosa does not apply equally to all species, but is constrained in some by the  $\Delta^4$  block to androstenedione formation in the theca. In these species, the increase in 3 $\beta$ -HSD expression with luteinization (Doody *et al.*, 1990b; Couet *et al.*, 1990; Rogers, 1990; Voss and Fortune, 1993a; Jeungel *et al.*, 1994) may be as important as the decline in thecal CYP17 in bringing about the decrease in follicular androgen and estrogen synthesis that precedes ovulation. In effect, a higher ratio of CYP17:3 $\beta$ -HSD in the primate and ruminant follicle favors  $\Delta^5$  metabolism, androgen, and thereby estrogen, synthesis before ovulation, whereas a lower ratio favors progesterone synthesis by the corpus luteum. Equally consistent with the principles of regulation outlined for the adrenal gland, cytochrome b<sub>5</sub> expression is restricted to the theca interna (Conley and Mapes, unpublished observations). Thus, cytochrome b<sub>5</sub> expression probably further promotes 17,20-lyase activity, androgen and estrogen synthesis in the thecal compartment of the pre-ovulatory follicle.

The human and non-human primate corpus luteum presents an unusual case among mammals in that it also secretes a significant quantity of estrogen during the luteal phase of the cycle. In fact, the levels of CYP19 expression are much higher in the human CL than in the follicle (Doody *et al.*, 1990b). Evidence has been reported to suggest that a similar separation of CYP17 and CYP19 exist in the primate corpus luteum (Sanders and Stouffer, 1997). This is based on higher CYP17 expression in cells around the periphery, and along vascular channels, of the CL whereas CYP19 is expressed in the centrally located, large luteal cells. The pattern of expression is not uniform however, but mosaic in appearance, suggesting heterogenous expression and differentiation among luteal cell types. Though these patterns of expression may be consistent with the above-mentioned "two-cell" hypothesis, these morphological studies require support from investigations of functional enzyme and steroid secretory activities. Cultured human luteinized granulosa cells secrete nanogram quantities of estrogen without detectable levels of CYP17 expression (Morán *et al.*, 2000). These data suggest that as low as CYP17 expression is in human luteinized



granulosa (Voutilainen *et al.*, 1986), it is adequate to support a substantial level of estrogen secretion *in vitro* without provision of androgen from other ovarian cell types.

### LESSONS FROM NATURAL MUTATIONS AND KNOCKOUTS

Genetic null mutations or gene knockouts have provided useful information, confirming suspected functions, suggesting new ones, and identifying alternative pathways or adaptations. Recent studies have provided some of the best examples. The discovery of the StAR protein (Clark *et al.*, 1994; Stocco, 1999) led to the realization that a great many patients with congenital lipoid adrenal hyperplasia suffer a defect in the gene encoding it (Bose *et al.*, 1996; see also chapters 7 and 13). This has greatly advanced our understanding of this disease which was previously attributed to an elusive defect in CYP11A, but with considerable debate (Lin *et al.*, 1991b). It is now known, for instance, that pregnancy can be established in women that are heterozygous for a null mutation in StAR. One such patient has been monitored during a pregnancy involving a fetus that was homozygous for the null allele (Saenger *et al.*, 1995). Maternal levels of estriol were extremely low, which was consistent with a lack of fetal adrenal androgen secretion. The pregnancy proceeded to term however, and symptoms consistent with congenital adrenal hyperplasia were obvious in the neonate soon after birth. The maintenance of pregnancy in the absence of StAR was perhaps a surprising feature (Saenger *et al.*, 1995) because it was anticipated that such a defect would preclude placental progesterone production. The prenatal diagnosis of the condition, allowing an evaluation of fetal steroid hormone levels, provided invaluable information. Most steroids were low or undetectable, consistent with a lack of fetal adrenal steroid secretion that was specifically evidenced by the low maternal estriol concentrations. However, amniotic pregnenolone levels were one third those of an unaffected sibling, and progesterone levels were about half normal values. Thus, in light of the recognized defect in StAR, and the expected blockade of all steroidogenesis, the appearance of pregnenolone and progesterone in amniotic fluid suggests the existence of alternative mechanisms for the supply of cholesterol to the inner mitochondrial membrane. Subsequent studies suggested that the human placenta does not normally express StAR (Pollack *et al.*, 1997), additional evidence of StAR-independent steroidogenesis (Sugawara *et al.*, 1995). This is apparently not true of all mammals, because StAR expression has been detected in both the bovine and porcine placenta (Pilon *et al.*, 1997). A relatively low rate of progesterone production by the placenta of these species is supported largely by placental mass (Conley and Mason, 1990; Geisert and Conley, 1998). Nonetheless, it appears that while StAR supports the major portion of cholesterol transport to the mitochondria for steroidogenesis, other mechanisms that have yet to be properly defined provide non-StAR sources of substrate for CYP11A. Some of these are introduced in later chapters.

A few relevant questions, however, still remain. Firstly, if the human placenta lacks StAR expression, and placental progesterone secretion normally proceeds without it by utilizing an alternative mechanism of cholesterol transport, why were there lowered pregnane levels in amniotic fluid of the above mentioned lipoidal CAH patient? Apparently, the progesterone secretory capacity of the placenta of this patient was not examined, but it would be interesting to know how it compared with normal placental tissue in this regard.

Secondly, Saenger *et al.* (1995) failed to find a difference in steroid hormone response to ACTH challenge of heterozygous carrier parents in the case. Similar results were reported for the StAR knockout mouse (Caron *et al.*, 1997), and both sets of observations have important implications for the regulation of adrenal steroidogenesis. Unfortunately, it is not known if StAR expression and activity are sensitive to gene dose effects in either case, although such dose effects have been demonstrated for other steroidogenic enzymes (White, 1994 and below). If such is the case for StAR, then it could again be argued that a decrease in StAR without an effect on adrenal steroid output indicates that it may not be the rate limiting step, or at least the sole limiting factor of adrenal corticoid synthesis. Clearly further studies are still needed in this area.

The discovery of a strain of rabbits with a congenital deficiency in P450scc (Pang *et al.*, 1992; Yang *et al.*, 1993) is a notable example of how natural or experimental gene knockouts might provide more useful information on the *regulation* of steroidogenesis than has been forthcoming to date from results of studies involving, almost exclusively, null mutants. Two points are instructive. It has been concluded that, in contrast to the human circumstance, defects in CYP11A are viable only because pregnancy in the rabbit is dependent on maternal luteal, not conceptus placental, progesterone synthesis (Miller, 1998). This contrasts the proposal that a complete deficiency of CYP11A, or of any of the proteins involved in placental progesterone secretion (adrenodoxin, adrenodoxin reductase, 3 $\beta$ -HSD type I), is incompatible with the maintenance of human pregnancies to term (Miller, 1998). None have yet been recognized. Such considerations of species differences in basic biology are certainly critical and all too often over-looked. However, there is a second important lesson from this animal model of more relevance to the regulation of steroidogenesis. The survival to term of rabbit fetuses completely lacking CYP11A is not surprising, given that symptoms of glucocorticoid deficiency develop almost immediately post-partum. However, data reported for those rabbits that are heterozygous for the defect are striking. These rabbits exhibit a gene dose effect with respect to the CYP11A expression, at least in adrenal tissues (Iwamoto *et al.*, 1994). Heterozygotes express CYP11A at half the level of that seen in normal contemporaries. Despite halving CYP11A, the heterozygotes have normal baseline and stimulated corticosterone levels. Data for progesterone concentrations during pregnancy in the heterozygous dam were not reported, but might be predicted to show that luteal progesterone secretion was not affected by halving levels of CYP11A in the corpora lutea of pregnancy. Similarly, it would be interesting to know if basal or stimulated testicular androgen secretion was within normal limits in heterozygous males. These data would support the conclusion that reduced CYP11A expression does not limit corticoid secretion by the rabbit adrenal. Perhaps StAR expression does limit steroidogenesis, but similar careful and more complete studies on the heterozygous siblings of the StAR knockout patients (Saenger *et al.*, 1995) and mice (Caron *et al.*, 1997) might yield the necessary data to confirm such predictions.

Unfortunately, the complete absence of a gene or a gene product contributes relatively little to an understanding of the regulation of steroidogenesis. A complete knockout of any gene in the pathway, unless an alternative biosynthetic route or source of substrate exists, will result in complete loss of the end product. If it is an essential steroid, the phenotype can be devastating, and in many cases it is. Even if it is not a lethal event, the contribution of the

gene product is missing during development and the consequences of this on function later in life are hard to predict if compensatory mechanisms are activated. Though one can argue that complete absence of certain genes may be incompatible with survival *in utero* (CYP11A, adrenodoxin, adrenodoxin reductase, 3 $\beta$ -HSD type I, as explained above), it does not preclude the existence of individuals heterozygous for these defects as in others already known. The existence of enzymatic defects in the heterozygous state may tell us even more than null mutants about the regulation of steroidogenesis, if the levels of enzyme, or activity more importantly, were known. Not only is the gene product at least present during development, but also additional knowledge of the basal and stimulated hormone levels would indicate the rate limiting potential for that particular component. There has been considerable interest in the use of hormonal assays in conjunction with genetic analyses to diagnose heterozygous defects in steroidogenic enzymes (Nayak *et al.*, 1998; Witchel and Lee, 1998). Unfortunately, gene dosage effects on enzyme expression and activity are not known. Moreover, some of these defects are not totally inactivating, and partial catalytic activity of one allele becomes an additional complicating factor. The expression of genes encoding for enzymes with partial function are more difficult to recognize in populations but such cases provide potentially much more physiological information pertinent to regulation. Studies conducted on patients with natural mutations of CYP21 are an excellent case in point. *In vitro* expression of the mutant enzymes demonstrated that reduced catalytic activity was not associated with detectable glucocorticoid deficiency until over 50% of the activity of the wild type was lost. Nor was aldosterone synthesis influenced until over 90% of wild-type activity was lost (Tusie-Luna *et al.*, 1990; Strachan and White, 1991; New, 1995). This suggests that *in vivo*, CYP21 functions at levels considerably higher than those that might otherwise limit corticoid secretion. In other words, CYP21 is unlikely to represent a point of regulation of glucocorticoid or mineralocorticoid synthesis under normal physiological conditions in humans. Few other examples exist that have been studied so completely.

#### LESSONS FROM PREGNANCY AND PARTURITION

A clear, anatomical separation of androgen and estrogen synthesis is also seen in a number of cases during pregnancy (Geisert and Conley, 1998). Estrogen is a major secretory product of the healthy human conceptus, and the expression of CYP19 in the human placenta is exceptional (Simpson and MacDonald, 1981) in comparison to other primate and mammalian species (Jurke *et al.*, 1998). However, neither CYP17 expression nor activity is detectable in human placenta, and androgens aromatized *in situ* have their origins in the fetal zone of the developing fetal adrenal gland (Simpson and MacDonald, 1981), as noted previously. Therefore, rather than a co-ordinated "two-cell" system, two separate fetal organs participate in estrogen synthesis during pregnancy. Estrogen synthesis follows a similar scheme in the pregnant mare except that androgens derived from the fetal gonads provide the substrate for aromatization in the placenta (Pashen and Allen, 1979). The rat and mouse placenta presents yet another strategy for estrogen synthesis during pregnancy. Unlike the human and equine placentas, the rodent placenta expresses CYP17 at increasing levels during the second half of gestation (Durkee *et al.*, 1992). However, the rodent placenta does not express CYP19, and placental androgens that escape metabolism in the

uterus are aromatized in the maternal corpora lutea (Hickey *et al.*, 1988). The need to carefully regulate estrogen synthesis in the pregnant mouse has become particularly clear as a result of studies in which 5 $\alpha$ -reductase type I was genetically knocked out (Mahendroo *et al.*, 1996). Surprisingly, this mutation induced a high rate of fetal death that has been attributed to the effects of an over-production of estrogen resulting from a decrease in the rate of androgen metabolism in the placenta (Mahendroo *et al.*, 1997). Additional problems are encountered at parturition as a result of a lack of cervical ripening (Mahendroo and Russell, 1999).

Estrogens are produced in relatively modest amounts during pregnancy in most other species and physiological effects are probably restricted to the immediate uterine environment (Geisert and Conley, 1998). The modest rates of estrogen synthesis by conceptus tissues seem to reflect the generally low levels of CYP17 expression typical of most mammalian placentas relative to those of the rat and mouse. There are two noteworthy exceptions to this general rule. In the cow (Conley *et al.*, 1992) there is a transient rise in placental CYP17 expression coinciding with organization of the fetal adrenal gland and possibly an early burst of fetal adrenal cortisol synthesis, also seen in the fetal sheep (Wintour *et al.*, 1995). The physiological relevance is unknown but it coincides with susceptibility to certain intra-uterine pathogens that may be a reflection of steroid induced alterations in uterine immune response. However, there is a more pronounced and physiologically relevant rise in placental CYP17 expression that is induced by the burst of fetal adrenal cortisol secretion associated with induction of parturition (France *et al.*, 1988). It is of interest to note that not only does the rise in fetal adrenal cortisol appear to be due to a pre-term rise in pituitary ACTH (Norwitz *et al.*, 1999) but studies in fetal ovine adrenal suggest that the rise in CYP17 expression only occurs after a dramatic fall in fetal adrenal AT1-R expression at that time (Coulter *et al.*, 2000). Such a finding is consistent once again with the aforementioned proposed role of AII as an important attenuator/modulator of CYP17 expression. The rise in CYP17, and the likely decreased ratio of 3 $\beta$ -HSD:CYP17 probably promotes androgen synthesis by the mechanisms described above. An accompanying increase in placental CYP19 expression (France *et al.*, 1988) also facilitates the final rise in estrogen secretion that helps to co-ordinate events leading to the successful maternal preparation for parturition and lactation. It is worth noting here that many authors have erroneously interpreted these events as switching placental steroid synthesis from progesterone to estrogen. Review of the evidence cited above that in ruminant species, as in human tissues, progesterone is a poor substrate for androgen synthesis (Fevold *et al.*, 1989), if at all, will obviate this fallacy. The equally important consequence of realizing the distinction is that the increase in placental CYP17 has little to do with the withdrawal of progesterone in these species which is brought about by an increase in progesterone metabolism (Geisert and Conley, 1998), perhaps in large measure. This would be expected to involve the induction of metabolic enzymes of the SDH or ARK type which future studies may reveal. Regardless, changes in the cellular expression of an array of enzymes within chorioallantoic cells appears to maintain a critical balance the local steroid environment in the uterus that changes to accommodate the evolving physiological needs of pregnancy. Similar principles are likely to hold answers of relevance to the role of steroid hormone synthesis and metabolism during human pregnancy.

## CONCLUSIONS

Clearly steroidogenesis is a complex process whereby only a few enzymes and accessory proteins are needed to produce an array of products of biological importance. The complexity of steroidogenesis and its regulation through tissue-specific control of relative enzyme levels, accessory proteins, subcellular compartmentalization, control of electron supply, possibly phosphorylation and selective inhibition by soluble gases is what give this important endocrine system the power and flexibility to meet the evolving needs of the host. Examination of species differences really teaches us that regulation actually can occur at each and every level, the extent of which depends on the species and tissue in question. The power of comparing data from different species is therefore enormous but equally extrapolation of assumptions from one species to another are potentially misleading. With only a very few members of the gene families known, further studies in this area will surely be fruitful in advancing our understanding the role of steroids in growth, development and normal body function, and the mechanisms by which this is achieved at a cellular and molecular level.

## REFERENCES

- Abbarszade, I.G., Clarke, T.R., Park, C.J. and Payne, A.H. (1995) The mouse  $3\beta$ -hydroxysteroid dehydrogenase multigene family includes 2 functionally distinct groups of proteins. *Mol. Endocrinol.* **9**, 1212–1222.
- Akhtar, M., Lee-Robichaud, P., Akhtar, M.E. and Wright, J.N. (1997) The impact of aromatase mechanism on other P450s. *J. Steroid Biochem. Molec. Biol.* **61**, 127–132.
- Anapliotou, M.L.G., Liparaki, M., Americanos, N., Goulandrakis, N. and Papioannou, D. (1994) Increased 17-OH-progesterone levels following hCG stimulation in men with idiopathic oligozoospermia and raised FSH. *Int. J. Androl.* **17**, 192–198.
- Andersson, S. and Moghrabi, N. (1998)  $17\beta$ -hydroxysteroid dehydrogenase: physiological roles in health and disease. *Trends Endocrinol. Metab.* **9**, 265–270.
- Ariyoshi, N., Kim, Y.C., Artmenko, I., Bhattacharyya, K. and Jefcoate, C.R. (1998) Characterization of the rat StAR gene that encodes the predominant 3.5-kilobase pair mRNA. *J. Biol. Chem.* **273**, 7610–7619.
- Armstrong, D.T., Weiss, T.J., Selstam, G. and Seamark, R.F. (1981) Hormonal and cellular interactions in follicular steroid biosynthesis by the sheep ovary. *J. Reprod. Fertil.* **30** (Suppl.), 143–154.
- Artemenko, I.P., Zhao, D., Hales, D.B., Hales, K.H. and Jefcoate, C.R. (2001) Mitochondrial processing of newly synthesized steroidogenic gene regulatory protein (StAR), but not total StAR, mediates cholesterol transfer to cytochrome P450 side chain cleavage enzyme in adrenal cells. *J. Biol. Chem.* **276**, 46583–46596.
- Auchus, R.J., Lee, T.C. and Miller, W.L. (1998) Cytochrome  $b_5$  augments the 17,20-lyase activity of human P450c17 without direct electron transfer. *J. Biol. Chem.* **273**, 3158–3165.
- Baker, M.E. (1991) Genealogy of regulation of human sex and adrenal function, prostaglandin action, snapdragon and petunia flower colors, antibiotics, and nitrogen fixation: functional diversity from two ancestral dehydrogenases. *Steroids* **56**, 354–360.
- Baker, M.E. (1996) Unusual evolution of  $11\beta$ - and  $17\beta$ -hydroxy steroid and retinol dehydrogenases. *Bioessays* **18**, 63–70.

- Barnes, H.J., Arlotto, M.P. and Waterman, M.R. (1991) Expression and enzymatic activity of recombinant cytochrome P450 17 $\alpha$ -hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5597–5601.
- Baulieu, E.E., Fabre-Jung, I. and Huis int Veld, L.G. (1967) Dehydroepiandrosterone sulfate: a secretory product of the boar testis. *Endocrinology* **81**, 34–38.
- Belanger, B., Caron, S., Belanger, A. and Dupont, A. (1990) Steroid fatty acid esters in adrenals and plasma: Effects of ACTH. *J. Endocrinol.* **127**, 505–511.
- Biason-Lauber, A., Leiberman, E. and Zachmann, M. (1997) A single amino acid substitution in the putative redox partner-binding site of P450c17 as a cause of isolated 17,20-lyase deficiency. *J. Clin. Endocrinol. Metab.* **82**, 3807–3812.
- Bird, I.M., Magness, R.R., Mason, J.I. and Rainey, W.E. (1992) Angiotensin II acts via the type-1 receptor to inhibit 17 $\alpha$ -hydroxylase cytochrome P450 expression in ovine adrenocortical cells. *Endocrinology* **130**, 3113–3121.
- Bird, I.M., Mason, J.I. and Rainey, W.E. (1998) Battle of the kinases: integration of adrenal responses to cAMP, DG and Ca<sup>2+</sup> at the level of steroidogenic cytochromes P450 and 3 $\beta$ -HSD expression in H295R cells. *Endocr. Res.* **24**, 345–354.
- Bird, I.M., Mathis, J.M., Mason, J.I. and Rainey, W.E. (1995) Ca<sup>2+</sup> regulated expression of steroid hydroxylases in H295R human adrenocortical cells. *Endocrinology* **136**, 5677–5684.
- Bird, I.M., Pasquarette, M.M., Rainey, W.E. and Mason, J.I. (1996a) Differential control of 17 $\alpha$ -hydroxylase and 3 $\beta$ -hydroxy steroid dehydrogenase expression in human adrenocortical H295R cells. *J. Clin. Endocrinol. Metab.* **81**, 2171–2178.
- Bird, I.M., Walker, S.W. and Williams, B.C. (1990) Review: agonist stimulation of phosphoinositide turnover in the adrenal cortex and its relationship to steroidogenesis. *J. Mol. Endocrinol.* **5**, 191–209.
- Bird, I.M., Zheng, J., Corbin, C.J., Magness, R.R. and Conley, A.J. (1996b) Immunohistochemical analysis of AT1 receptor versus P450c17 and 3 $\beta$ -HSD expression in ovine adrenals. *Endocr. Res.* **22**, 349–354.
- Bose, H.S., Sugawara, T., Strauss, J.F. and Miller, W.L. (1996) The pathophysiology and genetics of congenital adrenal hyperplasia. *New Engl. J. Med.* **335**, 1870–1878.
- Bosu, W.T.K., Holmdahl, T.H., Johansson, E.D.B. and Gemzell, C. (1972) Peripheral plasma levels of oestrogens, progesterone and 17 $\alpha$ -hydroxyprogesterone during the menstrual cycle of the rhesus monkey. *Acta Endocrinol.* **71**, 755–764.
- Brock, B.J. and Waterman, M.R. (1999) Biochemical differences between rat and human cytochrome P450c17 support the different steroidogenic needs of these two species. *Biochemistry* **38**, 1598–1606.
- Caron, K.M., Soo, S.-C., Wetsel, W.C., Stocco, D.M., Clark, B.J. and Parker, K.L. (1997) Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipid adrenal hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11540–11545.
- Channing, C.P. and Kammerman, S. (1974) Binding of gonadotropins to ovarian cells. *Biol. Reprod.* **10**, 179–198.
- Cherradi, N., Defaye, G. and Chambaz, E.M. (1994) Characterization of the 3 $\beta$ -hydroxy steroid dehydrogenase activity associated with bovine adrenal cortical mitochondria. *Endocrinology* **134**, 1358–1364.
- Choi, I., Troyer, D.L., Cornwall, D.L., Kirby-Dobbels, K.R., Collante, W.R. and Simmen, F.A. (1997) Closely related genes encode developmental and tissue isoforms of porcine cytochrome P450 aromatase. *DNA Cell. Biol.* **16**, 769–777.
- Clark, B.J., Wells, J., King, S.R. and Stocco, D.M. (1994) The purification, cloning, and expression of a novel LH-induced mitochondrial protein in MA-10 mouse Leydig tumor cells:

- Characterization of the steroidogenic acute regulatory (StAR) protein . *J. Biol. Chem.* **269**, 28314–28322.
- Conley, A.J. and Bird, I.M. (1997) The role of P450 17 $\alpha$ -hydroxylase and 3 $\beta$ -hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the  $\Delta^4$  and  $\Delta^5$  pathways of steroidogenesis. *Biol. Reprod.* **56**, 789–799.
- Conley, A.J., Corbin C.J., Hinshelwood, M.M., Lui, Z., Simpson, E.R., Ford, J.J. and Harada, N. (1996) Functional aromatase expression in porcine adrenal gland and testis. *Biol. Reprod.* **54**, 497–505.
- Conley, A.J., Corbin, C.J., Smith, T., Hinshelwood, M.M., Liu, Z. and Simpson, E.R. (1997) Porcine aromatases: studies on tissue-specific, functionally distinct isozymes from a single gene? *J. Steroid Biochem. Molec. Biol.* **61**, 407–413.
- Conley, A.J., Head, J.R., Stirling, D., Simpson, E.R. and Mason, J.I. (1992) Expression of steroidogenic enzymes in bovine placenta and fetal adrenals throughout gestation. *Endocrinology* **130**, 2641–2650.
- Conley, A.J., Howard, H.J., Slinger, W.D. and Ford, J.J. (1994) Steroidogenesis in the preovulatory porcine follicle. *Biol. Reprod.* **51**, 655–661.
- Conley, A.J., Kaminski, M.A., Dubowsky, S., Jablonka-Shariff, A., Redmer, D. and Reynolds, L.P. (1995) Immunohistochemical localization of 3 $\beta$ -hydroxy steroid dehydrogenase and P450 17 $\alpha$ -hydroxylase during follicular and luteal development in pigs, sheep and cattle. *Biol. Reprod.* **52**, 1081–1094.
- Conley, A.J. and Mason, J.I. (1990) Placental steroid hormones. *Bailliere's Clin. Endocrinol. Metab.* **4**, 249–272.
- Conley, A.J. and Mason, J.I. (1993) Steroidogenic enzymes. In: *Molecular Aspects of Placenta and Fetal Membrane Autocoids*, G.E.Rice and S.P.Brennecke (eds), CRC Press, pp. 1–25.
- Conley, A.J., Rainey, W.E. and Mason J.I. (1994) Ontogeny of steroidogenic enzyme expression in the porcine conceptus. *J. Mol. Endocrinol.* **12**, 155–165.
- Conley, A.J. and Walters, K.W. (1998) Aromatization. In: *Encyclopedia of Reproduction*, E.Knobil & J.D.Neill (eds), Academic Press, NY, USA (In press).
- Corbin, C.J., Khalil, M.W. and Conley, A.J. (1995) Functional ovarian and placental isoforms of porcine aromatase. *Mol. Cell. Endocrinol.* **113**, 29–37.
- Corbin, C.J., Trant, J.M., Walters, K.W. and Conley, A.J. (1999) Changes in testosterone metabolism associated with the evolution of placental and gonadal isozymes of porcine aromatase cytochrome P450. *Endocrinology* **140**, 5202–5210.
- Couet, J., Martel, C., Dupont, E., Luu-The, V., Sirard, M.-A., Zhao, H.-F., Pelletier, G. and Labrie, F. (1990) Changes in 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase messenger ribonucleic acid, activity and protein levels during the bovine estrous cycle in the bovine ovary. *Endocrinology* **127**, 2141–2148.
- Coulter, C.L., Myers, D.A., Nathanielsz, P.W. and Bird, I.M. (2000) Ontogeny of Angiotensin II Type-1 receptor (AT<sub>1</sub>R) and cytochrome P450c11 (CYP11B) in the sheep adrenal gland. *Biol. Reprod.* **62**, 714–719.
- Cutler, G.B., Glenn, M., Bush, M., Hodgen, G.D., Graham, C.E. and Loriaux, D.L. (1978) Adrenarche: a survey of rodents, domestic animals, and primates. *Endocrinology* **103**, 2112–2118.
- Daguet, M.-C. (1979) Increase of follicle cell LH binding and changes in the LH level of follicular fluid during the preovulatory period in the sow. *Ann. Biol. Anim. Bioch. Biophys.* **19**, 1655–1667.
- Del Punta, K., Charreau, E.H. and Pignataro, O.P. (1996) Nitric oxide inhibits Leydig cell steroidogenesis. *Endocrinology* **137**, 5337–5343.

- Doody, K.M., Carr, B.R., Rainey, W.R., Byrd, W., Murry, B.A., Strickler, R.C., Thomas, J.L. and Mason, J.I. (1990a) 3 $\beta$ -hydroxy steroid dehydrogenase/isomerase in the fetal zone and neocortex of the human fetal adrenal gland. *Endocrinology* **126**, 2487–2492.
- Doody, K., Lorence, M., Mason, J. and Simpson, E. (1990b) Expression of messenger ribonucleic acid species encoding steroidogenic enzymes in human follicles and corpora lutea throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.* **70**, 1041–1045.
- Duffy, D.M., Hess, D.L. and Stouffer, R.L. (1994) Acute administration of a 3 $\beta$ -hydroxy steroid dehydrogenase inhibitor to rhesus monkeys at the midluteal phase of the menstrual cycle: evidence for possible autocrine regulation of the primate corpus luteum by progesterone. *J. Clin. Endocrinol. Metab.* **79**, 1587–1594.
- Duffy, D.M., Molskness, T.A. and Stouffer, R.L. (1996) Progesterone receptor messenger ribonucleic acid and protein in luteinized granulosa cells of Rhesus monkeys are regulated *in vitro* by gonadotropins and steroids. *Biol. Reprod.* **54**, 888–895.
- Durkee, T.J., McClean M.P., Hales, D.B., Payne, A.H., Waterman, M.R., Khan, I. and Gibori, G. (1992) P450 17 $\alpha$  and P450scc gene expression and regulation in the rat placenta. *Endocrinology* **130**, 1309–1317.
- El-Migdadi, F., Gallant, S. and Brownie, A.C. (1995) Sex differences in the steroidogenic and respiratory electron transport chains in the rat adrenal cortex. *Endocr. Res.* **21**, 109–114.
- Engelbrecht, Y. and Swart, P. (2000) Adrenal function in Angora goats: a comparative study of adrenal steroidogenesis in Angora goats, Boer goats and Merino sheep. *J. Anim. Sci.* **78**, 1036–1046.
- Erickson, G.F., Magoffin, D.A., Dyer, C.A. and Hofeditz, C. (1985) The ovarian androgen producing cells: a review of structure/function relationships. *Endocr. Rev.* **6**, 371–399.
- Escobar-Morreale, H., Pazos, F., Potau, N., Garcia-Robles, R., Sancho, J.M. and Varela, C. (1994) Ovarian suppression with triptorelin and adrenal stimulation with adrenocorticotropin in functional hyperandrogenism: role of adrenal and ovarian cytochrome P450c17 $\alpha$ . *Fertil. Steril.* **62**, 521–530.
- Estabrook, R.W., Cooper, D.Y. and Rosenthal, O. (1963) The light-reversible carbon monoxide inhibition of the steroid C-21 hydroxylation system of the adrenal cortex. *Biochem. Zeit.* **338**, 741–755.
- Estabrook, R.W., Franklin, M.R., Cohen, B., Shigamatzu A. and Hildebrandt, A.G. (1971) Influence of hepatic microsomal mixed function oxidation reactions on cellular metabolic control. *Metabolism* **20**, 187–199.
- Evans, G., Dobias, M., King, G.J. and Armstrong, D.T. (1981) Estrogen, androgen, and progesterone biosynthesis by theca and granulosa of preovulatory follicles in the pig. *Biol. Reprod.* **25**, 673–682.
- Falck, B. (1959) Site of production of oestrogen in rat ovary as studied in micro-transplants. *Acta Physiol. Scand.* **47** (Suppl. 163), 1–101.
- Fevold, H.R. (1967) Regulation of the adrenal cortex secretory pattern by adrenocorticotrophin. *Science* **156**, 1753–1755.
- Fevold, H.R., Lorence, M.C., McCarthy, J.L., Trant, J.M., Kagimoto, M., Waterman, M.R. and Mason, J.I. (1989) Rat P450<sub>17 $\alpha$</sub>  from testis: Characterization of a full-length cDNA encoding a unique steroid hydroxylase capable of catalyzing both  $\Delta^4$ - and  $\Delta^5$ -steroid-17,20-lyase reactions. *Mol. Endocrinol.* **3**, 968–975.
- Fevold, H.R., Wilson, P.L. and Slanina, S.M. (1978) ACTH-stimulated rabbit adrenal 17 $\alpha$ -hydroxylase kinetic properties and a comparison with those of 3 $\beta$ -hydroxysteroid dehydrogenase. *J. Steroid Biochem.* **9**, 1033–1041.



- Fortune, J.E. (1994) Ovarian follicular growth and development in mammals. *Biol. Reprod.* **50**, 225–232.
- Foxcroft, G.R. and Hunter M.G. (1985) Basic physiology of follicular maturation in the pig. *J. Reprod. Fert.* **33** (Suppl.), 1–19.
- France, J.T., Magness, R.R., Murry, B.A., Rosenfeld, C.R. and Mason, J.I. (1988) The regulation of ovine placental steroid 17-hydroxylase and aromatase by glucocorticoid. *Mol. Endocrinol.* **2**, 193–199.
- Gaiani, R., Chiesa, F., Mattioli, M., Nannetti, G. and Galeati, G. (1984) Androstenedione and testosterone concentrations in plasma and milk of the cow throughout pregnancy. *J. Reprod. Fertil.* **70**, 55–59.
- Garrett, W.M. and Guthrie, H.D. (1996) Expression of androgen receptors and steroidogenic enzymes in relation to follicular growth and atresia following ovulation in pigs. *Biol. Reprod.* **55**, 949–955.
- Garrett, W.M. and Guthrie, H.D. (1997) Steroidogenic enzyme expression during preovulatory follicular maturation in pigs. *Biol. Reprod.* **56**, 1424–1431.
- Geisert, R.D. and Conley, A.J. (1998) Secretion and metabolism of steroids in subprimate mammals during pregnancy. In: P.M.Conn (ed.), *Endocrinology of Pregnancy*, The Humana Press, Inc., Totowa, NJ, pp. 291–318.
- Geller, D.H., Auchus, R.J., Mendonca, B.B. and Miller, W.L. (1997) The genetic and functional basis of isolated 17,20-lyase deficiency. *Nat. Genet.* **17**, 201–205.
- Geller, D.H., Auchus, R.J. and Miller, W.L. (1999) P450c17 mutations R347H and R358Q selectively disrupt 17,20-lyase activity by disrupting interactions with P450 oxidoreductase and cytochrome b<sub>5</sub>. *Mol. Endocrinol.* **13**, 167–175.
- Giordano, S.J., Kaftory, A. and Steggle, A.W. (1994) A splicing mutation in the cytochrome b<sub>5</sub> gene from a patient with congenital methemoglobinemia and pseudohermaphroditism. *Hum. Genet.* **93**, 568–570.
- Graham, S.E. and Peterson, J.A. (1999) How similar are the P450s and what can they teach us? *Arch. Biochem. Biophys.* **369**, 24–29.
- Greenwald, G.S. and Roy, S.K. (1994) Follicular development and its control. In: *The Physiology of Reproduction*, Vol. 1, E.Knobel and J.D.Neill (eds), Raven Press, New York, pp. 629–724.
- Guengerich, F.P. and Macdonald, T.L. (1990) Mechanisms of cytochrome P-450 catalysis. *FASEB J.* **4**, 2453–2459.
- Hall, P.F. (1991) Cytochrome P-450 C<sub>21sc</sub>: one enzyme with two actions—hydroxylase and lyase. *J. Steroid Biochem. Molec. Biol.* **40**, 527–532.
- Haney, A.F. and Schomberg, D.W. (1981) Estrogen and progesterone production by developing porcine follicles *in vitro*: Evidence for estrogen formation by theca. *Endocrinology* **109**, 971–977.
- Hanke, C.J., Drewett, J.G., Myers, C.R. and Campbell, W.B. (1998) Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. *Endocrinology* **139**, 4053–4060.
- Hanukoglu, I. (1992) Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. *J. Steroid Biochem. Molec. Biol.* **43**, 779–804.
- Hanukoglu, I. and Hanukoglu, Z. (1986) Stoichiometry of mitochondrial cytochromes P-450, adrenodoxin and adrenodoxin reductase in adrenal cortex and corpus luteum: implications for membrane organization and gene regulation. *Eur. J. Biochem.* **157**, 27–31.
- Hanukoglu, I. and Rapaport, R. (1995) Routes and regulation of NADPH production in Steroidogenic mitochondria. *Endocr. Res.* **21**, 231–241.
- Haynes, R.C. (1975) Theories on the mode of action of ACTH in stimulating secretory activity of the adrenal cortex. In: *Handbook of Physiology, Endocrinology*, Vol. 6, American Physiological Society, Washington DC, pp. 69–76.

- Hickey, G.J., Chen, S.A., Besman, M.J., Shivery, J.E., Hall, P.F., Gaddy-Kurten, D. and Richards, J.S. (1988) Hormonal regulation, tissue distribution, and content of aromatase cytochrome P450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: relationship to estradiol biosynthesis. *Endocrinology* **122**, 1426–1436.
- Hillier, S.G., Whitelaw, P.F. and Smyth, C.D. (1994) Follicular oestrogen synthesis: the “two-cell, two-gonadotropin” model revisited. *Mol. Cell. Endocrinol.* **100**, 51–54.
- Hinshelwood, M.M., Demeter-Arlotto, M., Means, G.D. and Simpson, E.R. (1993) Molecular biology of genes encoding Steroidogenic enzymes in the ovary. In: *The Ovary*, E.Y. Adashi and P.C.K. Leung (eds), Raven Press, NY, pp. 165–183.
- Hobkirk, R. (1993) Steroid sulfation. *Trends Endocrinol. Metab.* **4**, 69–74.
- Holmdahl, T.H. and Johansson, E.D.B. (1972) Peripheral plasma levels of 17 $\alpha$ -hydroxyprogesterone, progesterone and oestradiol during normal menstrual cycles in women. *Acta Endocrinol.* **71**, 743–754.
- Hornsby, P.J. and Aldern, K.A. (1984) Steroidogenic enzyme activities in cultured human definitive zone adrenocortical cells: Comparison with bovine adrenocortical cells and resultant differences in adrenal androgen synthesis. *J. Clin. Endocrinol. Metab.* **58**, 121–127.
- Hsueh, A.J.W., Adashi, E.Y., Jones, P.B.C. and Welsh, T.H. (1984) Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocr. Rev.* **5**, 76–127.
- Hunter, M.G. and Armstrong, D.T. (1987) Oestrogens inhibit steroid production by porcine thecal cells. *Mol. Cell. Endocrinol.* **50**, 273–279.
- Hunter, M.G., Biggs, C., Pickard, A.R. and Faillace, L.S. (1994) Differences in follicular aromatase activity between Meishan and Large-White hybrid gilts. *J. Reprod. Fertil.* **101**, 139–144.
- Imai, T., Globerman, H., Gertner, J.M., Kagawa, N. and Waterman, M.R. (1993) Expression and purification of functional human 17 $\alpha$ -hydroxylase/17,20 lyase (P450c17) in *Escherichia coli*. *J. Biol. Chem.* **268**, 19681–19689.
- Ishi-Ohba, H., Matsumura, R., Inano, H. and Tamaoki, B. (1984) Contribution of cytochrome b<sub>5</sub> to androgen synthesis in rat testicular microsomes. *J. Biochem.* **95**, 335–343.
- Iwamoto, K., Yang, X., Rogerson, P.M., Mason, J.L., Artwohl, J., Bolin, K., Klimah, P., Swart, P. and Pang, S. (1994) Evidence of a steroidogenic enzyme gene dose effect on adrenal gene expression in hereditary rabbit congenital adrenal hyperplasia. *Ped. Res.* **36**, 660–666.
- Jeungel, J.L., Guy, M.K., Tandeski, T.R., McGuire, W.J. and Niswender, G.D. (1994) Steady-state concentration of messenger ribonucleic acid encoding cytochrome P450 cholesterol side-chain cleavage and 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase in ovine corpora lutea during the estrous cycle. *Biol. Reprod.* **51**, 380–384.
- Jurke, M.H., Czekala, N.M., Jurke, S., Hagey, L.R., Lance, V.A., Conley, A.J. and Fitch-Snyder, H. (1998) Monitoring pregnancy in twinning Pygmy Loris (*Nycticebus pygmaeus*) using fecal estrogen metabolites. *Am. J. Prim.* **46**, 173–183.
- Kass, E.H., Hechter, O., Macchi, I.A. and Mou, T.W. (1954) Changes in patterns of secretion of corticosteroids in rabbits after prolonged treatment with ACTH. *Proc. Soc. Exp. Med. Biol.* **85**, 583–587.
- Katagiri, M., Kagawa, N. and Waterman, M.R. (1995) The role of cytochrome b<sub>5</sub> in the biosynthesis of androgens by human P450c17. *Arch. Biochem. Biophys.* **317**, 343–347.
- Katagiri, M., Suhara, K., Shiroo, M. and Fujimura, Y. (1982) Role of cytochrome b<sub>5</sub> in the cytochrome P-450 mediated C21-steroid 17, 20 lyase reaction. *Biochem. Biophys. Res. Commun.* **108**, 379–384.
- Kletzien, R.F., Harris, P.K. and Foellmi, L.A. (1994) Glucose-6-phosphate dehydrogenase: a “housekeeping” enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. *FASEB J.* **8**, 174–181.

- Kletzien, R.F., Prostko, C.R., Stumpo, D.J., McClung J.K. and Dreher, K.L. (1985) Molecular cloning of DNA sequences complementary to rat liver glucose-6-phosphate dehydrogenase mRNA: nutritional regulation of mRNA levels. *J. Biol. Chem.* **260**, 5621–5624.
- Kominami, S., Ogawa, N., Morimune, R., De-Ying, H. and Takemori, S. (1992) The role of cytochrome b<sub>5</sub> in adrenal microsomal steroidogenesis. *J. Steroid Biochem. Molec. Biol.* **42**, 57–64.
- Krozowski, Z. (1999) The 11 $\beta$ -hydroxysteroid dehydrogenases: Functions and physiological effects. *Mol. Cell. Endocrinol.* **151**, 212–227.
- Labrie, F. (1991) Intracrinology. *Mol. Cell. Endocrinol.* **78**, CII3–CII8.
- Labrie, F., Simard, J., Luu-The, V., Belanger, A. and Pelletier, G. (1992) Structure, function and tissue-specific gene expression of 3 $\beta$ -hydroxy steroid dehydrogenase/5-ene-4-ene isomerase enzymes in classical and peripheral intracrine tissues. *J. Steroid Biochem. Molec. Biol.* **43**, 805–826.
- Labrie, F., Simard, J., Luu-The, V., Pelletier, G., Belghmi, K. and Belanger, A. (1994) Structure, regulation and role of 3 $\beta$ -hydroxysteroid dehydrogenase, 17 $\beta$ -hydroxysteroid dehydrogenase and aromatase enzymes in the formation of sex steroids in classical and peripheral intracrine tissues. *Balliere's Clin. Endocrinol. Metab.* **8**, 451–474.
- Lambeth, J.D., Seybert, D.W., Lancaster, Jr. J.R., Salerno, J.C. and Kamin, H. (1982) Steroidogenic electron transport in adrenal cortex mitochondria. *Molec. Cell. Biochem.* **45**, 13–31.
- Lautincik, J., Kolodzieyski, L., Elias, V., Hyttel, P., Osawa, Y. and Sirotkin, A. (1994) Immunocytochemical localization of aromatase in the ovary of super-ovulated cattle, pigs and sheep. *Acta Vet. Scand.* **35**, 185–191.
- Lee-Robichaud, P., Wright, J.N., Akhtar, M. and Akhtar, M. (1995) Modulation of the activity of human 17 $\alpha$ -hydroxylase-17,20-lyase (CYP17) by cytochrome b<sub>5</sub>: endocrinological and mechanistic implications. *Biochem. J.* **308**, 901–908.
- Lieberman, S., Greenfield, N.J. and Wolfson, A. (1984) A heuristic proposal for understanding steroidogenic processes. *Endocr. Rev.* **5**, 128–148.
- Lin, D., Harikrishna, J.A., Moore, C.C.D., Jones, K.L. and Miller, W.L. (1991a) Missense mutation serine106→proline causes 17 $\alpha$ -hydroxylase deficiency. *J. Biol. Chem.* **266**, 15992–15998.
- Lin, D., Gitelman, S.E., Saenger, P. and Miller, W.L. (1991b) Normal genes for the cholesterol side chain cleavage enzyme, P450<sub>scc</sub>, in congenital adrenal hyperplasia. *J. Clin. Invest.* **88**, 1955–1962.
- Lin, M.T., Haksar, A. and Peron, F.G. (1974) The role of the Krebs cycle in the generation of intramitochondrial reducing equivalents for the 11 $\beta$ -hydroxylation of deoxycorticosterone in isolated rat adrenal cells. *Arch. Biochem. Biophys.* **164**, 429–439.
- Lobanov, N.A., Hensey, C.E., Usanov, S.A. and Azzi, A. (1993) Phosphorylation of cytochrome P-450 (scc) by protein kinase C-protective effects of adrenodoxin and cytochrome b(5). *BiochemRussia* **58**, 1118–1124.
- Lorence, M.C., Murry, B.A., Trant, J.M. and Mason, J.I. (1990) Human 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase from placenta: expression in non-steroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids. *Endocrinology* **126**, 2493–2498.
- Mahendroo, M.S., Cala, K.M., Landrum, D.P. and Russell, D.W. (1997) Fetal death in mice lacking 5- $\alpha$ -reductase type 1 caused by estrogen excess. *Mol. Endocrinol.* **11**, 917–927.
- Mahendroo, M.S., Cala, K.M. and Russell, D.W. (1996) 5 $\alpha$ -reduced androgens play a key role in murine parturition. *Mol. Endocrinol.* **10**, 380–392.
- Mahendroo, M.S. and Russell, D.W. (1999) Male and female isoenzymes of steroid 5 $\alpha$ -reductase. *Rev. Reprod.* **4**, 179–183.

- Mapes, S., Corbin, C.J., Tarantal, A. and Conley, A.J. (1999) The primate adrenal zona reticularis is defined by expression of cytochrome b<sub>5</sub>, 17 $\alpha$ -hydroxylase/17, 20-lyase cytochrome P450 (P450c17) and NADPH-cytochrome P450 reductase (reductase) but not 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase (3 $\beta$ -HSD) . *J. Clin. Endocrinol. Metab.* **84**, 3382–3385.
- Martel, C., Melner, M.H., Gagne, D., Simard, J. and Labrie, F. (1994) Widespread tissue distribution of steroid sulfatase, 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase (3 $\beta$ -HSD), 17 $\beta$ -HSD, 5 $\alpha$ -reductase and aromatase activities in the rhesus monkey. *Mol. Cell. Endocrinol.* **104**, 103–111.
- Martini, G. and Ursini, M.V. (1996) A new lease of life for an old enzyme. *BioEssays* **18**, 631–637.
- Mason, J.I., Ushijima, K., Doody, K.M., Nagai, K., Naville, D., Head, J.R., Milewich, L., Rainey, W.E. and Ralph, M.M. (1993) Regulation of expression of the 3 $\beta$ -hydroxysteroid dehydrogenases of human placenta and fetal adrenal. *J. Steroid Biochem. Molec. Biol.* **47**, 151–159.
- Masuda, M., Kubota, T., Karnada, S. and Aso, T. (1997). Nitric oxide inhibits steroidogenesis in cultured porcine granulosa cells. *Mol. Hum. Reprod.* **3**, 285–292.
- Matsuda, H., Fujita, H., Ishimura, K. and Osawa, Y. (1984) Immunocytochemical localization of aromatase in ovaries of some rodents, cow and human. *Acta Histochem. Cytochem.* **17**, 311–322.
- McKerns, K.W. (1965) Additional studies on the mechanism of action of ACTH. *Can. J. Biochem.* **43**, 923–932.
- McNicol, A.M. (1992) The human adrenal gland. Aspects of structure, function and pathology. In: *The Adrenal Gland*, V.H.T.James (ed), Raven Press, NY, pp. 1–42.
- Meduri, G., VuHai, M.T., Jolivet, A., Takemori, S., Kominami, S., Driancourt, M.A. and Milgrom, E. (1996) Comparison of cellular distribution of LH receptors and steroidogenic enzymes in the porcine ovary. *J. Endocrinol.* **148**, 435–446.
- Meduri, G., Vuhai-Luuthi, M.T., Jolivet, A. and Milgrom, E. (1992) New functional zonation in the ovary as shown by immunohistochemistry of luteinizing hormone receptor. *J. Endocrinol.* **131**, 366–373.
- Mesiano, S., Coulter, C.L. and Jaffe, R.B. (1993) Localization of cytochrome P450 cholesterol side-chain cleavage, cytochrome P450 17 $\alpha$ -hydroxylase/17,20-lyase, and 3 $\beta$ -hydroxy steroid dehydrogenase isomerase steroidogenic enzymes in human and rhesus monkey fetal adrenal glands: Reappraisal of functional zonation. *J. Clin. Endocrinol. Metab.* **77**, 1184–1189.
- Miller, W.L. (1988) Molecular biology of steroid hormone biosynthesis. *Endocr. Rev.* **9**, 295–318.
- Miller, W.L. (1998b) Why nobody has P450scc (20, 22 desmolase) deficiency. *J. Clin. Endocrinol. Metab.* **83**, 1399–1400.
- Miller, W.L., Auchus, R.J. and Geller, D.H. (1997) The regulation of 17,20 lyase activity. *Steroids* **62**, 133–142.
- Morán, F.M., Conley, A.J., Corbin, C.J., Enan, E., VandeVoort, C., Overstreet, J.W. and Lasley, B.L. (2000) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) decreases estradiol production without altering the enzyme activity of cytochrome P450 aromatase of human luteinized granulosa cells *in vitro*. *Biol. Reprod.* **62**, 1102–1108.
- Mostl, E., Mostl, K., Choi, H.S., Drier, H.K., Stockl, W. and Bamberg, E. (1981) Plasma levels of androstenedione, epitestosterone, testosterone and oestrogens in cows at parturition. *J. Endocrinol.* **89**, 251–255.
- Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.G. (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**, 1248–1251.
- Namiki, M., Kitamura, M., Buczko, E. and Dufau, M.L. (1988) Rat testis P-450 17 $\alpha$  cDNA: the deduced amino acid sequence, expression and secondary structural configuration. *Biochem. Biophys. Res. Commun.* **157**, 705–712.

- Nathanielsz, P.W., Elsner, C., Magyar, D., Fridshal, D., Freeman, A. and Buster, J.E. (1982) Time trend analysis of plasma unconjugated and sulfoconjugated estrone and  $3\beta$ - $\Delta^5$ -steroids in fetal and maternal sheep plasma in relation to spontaneous parturition at term. *Endocrinology* **110**, 1402–1407.
- Nayak, S., Lee, P.A. and Witchel, S.F. (1998) Variants of the type II  $3\beta$ -hydroxysteroid dehydrogenase gene in children with premature pubic hair and hyperandrogenic adolescents. *Mol. Genet. Metab.* **64**, 184–192.
- Nelson, D.R. (1999) Cytochrome P450 and the individuality of species. *Arch. Biochem. Biophys.* **369**, 1–10.
- Nelson, D.R., Koymans, L., Kamataki T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C. and Nebert, D.W. (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6**, 1–42.
- New, M.I. (1995) Steroid 21-hydroxylase deficiency (congenital adrenal hyperplasia). *Am. J. Med.* **98**, 2S–8S.
- Norwitz, E.R., Robinson, J.N. and Challis, J.R.G. (1999) The control of labor. *New Engl. J. Med.* **341**, 660–666.
- Omura, T. and Sato, R. (1964) The carbon-monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **329**, 2370–2378.
- Orly, J. and Stocco, D.M. (1999) The role of steroidogenic acute regulatory (StAR) protein in female reproductive tissues. *Horm. Metab. Res.* **31**, 389–398.
- Ortiz de Montellano, P.R. (1989) Cytochrome P-450 catalysis: Radical intermediates and dehydrogenation reactions. *Trends Pharmacol. Sci.* **10**, 354–359.
- Pang, S., Yang, X., Wang, M., Tissot, R., Nino, M., Manaligod, J., Bullock, L.P. and Mason, J.I. (1992) Inherited congenital adrenal hyperplasia in the rabbit: Absent cholesterol side-chain cleavage cytochrome P450 gene expression. *Endocrinology* **131**, 181–186.
- Pashen, R.L. and Allen, W.R. (1979) The role of the fetal gonads and the placenta in steroid production, maintenance of pregnancy and parturition in the mare. *J. Reprod. Fertil.* **27** (Suppl.), 499–509.
- Peltoketo, H., Vihko, P. and Vihko, R. (1999) Regulation of estrogenic action: role of  $17\beta$ -hydroxysteroid dehydrogenases. *Vit. Horm.* **55**, 353–398.
- Peng, X.-R., Hsueh, A.J.W., LaPolta, P.S., Bjersing, L. and Ny, T. (1991) Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicular development and ovulation. *Endocrinology* **129**, 3200–3207.
- Penning, T.M. (1997) Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr. Rev.* **18**, 281–305.
- Penning, T.M. (1999) Molecular determinants of steroid recognition and catalysis in aldo-keto reductases. Lessons from  $3\alpha$ -hydroxysteroid dehydrogenase. *J. Steroid Biochem. Molec. Biol.* **69**, 211–225.
- Perkins, L.M. and Payne, A.H. (1988) Quantification of P450<sub>SCC</sub>, P450<sub>17 $\alpha$</sub> , and iron sulfur protein reductase in Leydig cells and adrenals of inbred strains of mice. *Endocrinology* **123**, 2675–2682.
- Pilon, N., Daneau, L., Brisson, C., Ethier, J.-F., Lussier, J.G. and Silversides, D.W. (1997) Porcine and bovine steroidogenic acute regulatory protein (StAR) gene expression during gestation. *Endocrinology* **138**, 1085–1091.
- Pollack, S.E., Furth, E.E., Kallen, C.B., Arakane, F., Kiriakidou, M., Kozarsky, K.F. and Strauss III, J.F. (1997) Localization of steroidogenic acute regulatory protein in human tissues. *J. Clin. Endocrinol. Metab.* **82**, 4243–4251.

- Porter, T.D. (1991) An unusual yet strongly conserved flavoprotein in bacteria and mammals. *TIBS*, **16**, 154–158.
- Quinn, S.J. and Williams, G.H. (1992) In: V.H.T.James (ed). The Adrenal Gland, 2nd edition, Raven Press Ltd, New York, pp. 159–189.
- Rainey, W.E., Naville, D. and Mason, J.I. (1991) Regulation of 3 $\beta$ -hydroxysteroid dehydrogenase in adrenocortical cells: Effects of angiotensin II and transforming growth factor beta. *Endocr. Res.* **17**, 281–296.
- Rainey, W.E., Naville, D., Saez, J.M., Carr, B.R., Byrd, W., Magness, R.R. and Mason, J.I. (1990) Transforming Growth factor- $\beta$  inhibits steroid 17 $\alpha$ -hydroxylase cytochrome P450 expression in ovine adrenocortical cells. *Endocrinology* **127**, 1910–1990.
- Rheume, E., Lachance, Y., Zhao, H.-F., Breton, N., Dumont, M., de Launoit, Y., Trudel, C., Luu-The, V., Simard, J. and Labrie, F. (1991) Structure and expression of a new complementary DNA encoding the almost exclusive 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in human adrenals and gonads. *Mol. Endocrinol.* **5**, 1147–1157.
- Richards, J.S. (1994) Hormonal control of gene expression in the ovary. *Endocr. Rev.* **15**, 725–751.
- Rodgers, R.J., Rodgers, H.F., Hall, P.F., Waterman, M.R. and Simpson, E.R. (1986) Immunolocalization of cholesterol side-chain cleavage cytochrome P-450 and 17 $\alpha$ -hydroxylase cytochrome P-450 in bovine ovarian follicles. *J. Reprod. Fertil.* **78**, 627–638.
- Rogers, R.J. (1990) Steroidogenic cytochrome P450 enzymes and ovarian steroidogenesis. *Reprod. Fertil. Dev.* **2**, 153–163.
- Rosenfield, R.L., Barnes, R.B. and Ehrmann, D.A. (1994) Studies of the nature of 17-hydroxyprogesterone hyperresponsiveness to gonadotropin-releasing hormone agonist challenge in functional hyperandrogenism. *J. Clin. Endocrinol. Metab.* **79**, 1686–1692.
- Russell, D.W., Berman, D.M., Bryant, J.T., Cala, K.M., Davis, D.L., Landrum, C.P., Prihoda, J.S., Silver, R.I., Thigpen, A.E. and Wigley, W.C. (1994) The molecular genetics of steroid 5 $\alpha$ -reductases. *Rec. Prog. Horm. Res.* **49**, 275–284.
- Saenger, P., Klonari, Z., Black, S.M., Compagnone, N., Mellon, S.H., Fleischer, A., Abrams, C.A.L., Shackleton, C.H.L. and Miller, W.L. (1995) Prenatal diagnosis of congenital adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **80**, 200–205.
- Sakai, Y., Yanase, T., Hara, T., Takayanagi, R., Haji, M. and Nawata, H. (1994) *In vitro* evidence for the regulation of 17, 20 lyase activity by cytochrome b<sub>5</sub> in adrenocortical adenomas from patients with Cushing's syndrome. *Clin. Endocrinol.* **40**, 205–209.
- Sanders, S.L. and Stouffer, R.L. (1997) Localization of Steroidogenic enzymes in macaque luteal tissue during the menstrual cycle and simulated early pregnancy: immunohistochemical evidence supporting the two-cell model for estrogen production in the primate corpus luteum. *Biol. Reprod.* **56**, 1077–1087.
- Sasano, H. (1994) Localization of Steroidogenic enzymes in adrenal cortex and its disorders. *Endocr. J.* **41**, 471–482.
- Sauer, L.A., Chapman, J.C. and Dauchy, R.T. (1994) Topology of 3 $\beta$ -hydroxy-5-ene-steroid dehydrogenase/delta 5-delta 4-isomerase in adrenal cortex mitochondria and microsomes. *Endocrinology* **134**, 751–759.
- Sazanov, L.A. and Jackson, J.B. (1994) Proton-translocating transhydrogenase and NAD- and NADP-linked isocitrate dehydrogenases operate in a substrate cycle which contributes to the fine regulation of the tricarboxylic acid cycle activity in mitochondria. *FEBS Lett.* **344**, 109–116.
- Sekihara, H. (1983) 19-Hydroxyandrostenedione: a potent hypertensinogenic steroid in man. *J. Steroid Biochem.* **19**, 353–358.
- Sethumadhavan, K. and Bellino, F.L. (1991) Human placental estrogen synthetase (aromatase). Effect of environment on the kinetics of protein-protein interactions and the production of 19-

- oxygenated androgen intermediates in the purified reconstituted cytochrome P450 system. *J. Steroid Biochem. Molec. Biol.* **39**, 381–394.
- Shen, A.L. and Casper, C.B. (1994) Protein and gene structure and regulation of NADPH-cytochrome P450 oxidoreductase. *Handbook Exp. Pharmacol.* **105**, 35–59.
- Shinzawa, K., Kominami, S. and Takemori, S. (1985) Studies on cytochrome P-450 (P-45017 $\alpha$ ,lyase) from guinea pig adrenal microsomes: dual function of a single enzyme and effect of cytochrome b<sub>5</sub>. *Biochim. Biophys. Acta* **833**, 151–160.
- Short, R.V. (1962) Steroids in the follicular fluid and the corpus luteum of the mare: A “two-cell type” theory of ovarian steroid synthesis. *J. Endocrinol.* **24**, 59–63.
- Siiteri, P.K. and Seron-Ferre, M. (1981) Some new thoughts on the fetoplacental unit and parturition in primates. In: *Fetal Endocrinology*, M.J.Novy and J.A.Resko (eds), Academic Press, NY, pp. 1–34.
- Simard, J., Melner, M.H., Breton, N., Low, K.G., Zhao, H-F., Periman, L.M. and Labrie, F. (1991) Characterization of macaque 3 $\beta$ -hydroxy-5-ene steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase: Structure and expression in Steroidogenic and peripheral tissues. *Mol. Cell. Endocrinol.* **75**, 101–110.
- Simpson, E.R. (1979) Cholesterol side-chain cleavage, cytochrome P450, and the control of steroidogenesis. *Mol. Cell. Endocrinol.* **13**, 213–227.
- Simpson, E.R. and MacDonald, P.C. (1981) Endocrine physiology of the placenta. *Annu. Rev. Physiol.* **43**, 163–188.
- Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., Mendelson, C.R. and Bulun, S.E. (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen synthesis. *Endocr. Rev.* **15**, 342–355.
- Smals, A.G.H., Pietters, G.F.F.M., Drayer, J.I.M., Boers, G.H.J., Benraad, T.J. and Kloppenvorg, P.W.C. (1980) Tamoxifen suppresses gonadotropin-induced 17 $\alpha$ -hydroxyprogesterone accumulation in normal men. *J. Clin. Endocrinol. Metab.* **51**, 1026–1029.
- Stewart, P.M. and Krozowski, Z.S. (1999) 11 $\beta$ -hydroxysteroid dehydrogenase. *Vit. Horm.* **57**, 249–333.
- Stocco, D.M. (1999) Steroidogenic acute regulatory protein. *Vit. Horm.* **55**, 399–441.
- Stocco, D.M., Wells, J. and Clark, B.J. (1993). The effects of hydrogen peroxide on steroidogenesis in mouse Leydig tumor cells. *Endocrinology* **133**, 2827–2832.
- Stone, B.A. and Seamark, R.F. (1985) Steroid hormones in uterine washings and in plasma of gilts between days 9 and 15 after oestrus and between days 9 and 15 after coitus. *J.Reprod. Fertil.* **75**, 209–221.
- Strachan, T. and White, P.C. (1991) Molecular pathology of steroid 21-hydroxylase deficiency. *J. Steroid Biochem. Molec. Biol.* **40**, 537–543.
- Strott, C.A., Yoshimi T., Ross, G.T. and Lipsett, M.B. (1969) Ovarian physiology: Relationship between plasma LH and steroidogenesis by the follicle and corpus luteum: Effect of hCG. *J. Clin. Endocrinol. Metab.* **29**, 1157–1167.
- Sugawara, T., Holt, J.A., Driscoll, D., Strauss III, J.F., Lin, D., Miller, W.L., Patterson, D., Clancy, K.P., Hart, I.M., Clark, B.J. and Stocco, D.M. (1995) Human Steroidogenic acute regulatory protein: Functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4778–4782.
- Swart, P., Englebrecht, Y., Bellstedt, D.U., de Villiers, C.A. and Dreesbeimdicke, C. (1995) The effect of cytochrome b<sub>5</sub> on progesterone metabolism in the ovine adrenal. *Endocr. Res.* **21**, 297–306.

- Swinney, D.C., Watson, D.M. and So, O.-Y. (1993) Accumulation of intermediates and isotopically sensitive enolization distinguish between aromatase (cytochrome P450 CYP19) from rat ovary and human placenta. *Arch. Biochem. Biophys.* **305**, 61–67.
- Tamaoki, B.-I. (1973) Steroidogenesis and cell structure: biochemical pursuit of sites of steroid synthesis. *J. Steroid Biochem.* **4**, 89–118.
- Tamura, T., Kitawaki, J., Yamamoto, T., Osawa, Y., Kominami, S., Takemori, S. and Okada, H. (1992) Immunolocalization of 17 $\alpha$ -hydroxylase/C 17-20 lyase and aromatase cytochromes P-450 in the human ovary during the menstrual cycle. *J. Endocrinol.* **135**, 589–595.
- Tremblay, Y., Belanger, A., Fleury, A., Beaudoin, C., Provost, P. and Martineau, I. (1995) Studies of the guinea pig adrenal cytochrome P450c17 cDNA. *Endocr. Res.* **21**, 495–507.
- Tremblay, Y., Fleury, A., Beaudoin, C., Vallee, M. and Belanger, A. (1994) Molecular cloning and expression of guinea pig cytochrome P450c17 cDNA (steroid 17 $\alpha$ -hydroxylase/17,20 lyase): tissue distribution, regulation, and substrate specificity of the expressed enzyme. *DNA Cell Biol.* **13**, 1199–1212.
- Tsubaki, M., Hiwatashi, A., Ichikawa, Y. and Hori, H. (1987) Electron paramagnetic resonance study of ferrous cytochrome P-450<sub>scc</sub>-nitric oxide complexes: effects of cholesterol and its analogues. *Biochemistry* **26**, 4527–4534.
- Tsubaki, M., Ichikawa, Y., Fujimoto, Y., Yu, N.T. and Hori, H. (1990) Active site of bovine adrenocortical cytochrome P-450(11) beta studied by resonance Raman and electron paramagnetic resonance spectroscopies: distinction from cytochrome P-450<sub>scc</sub>. *Biochemistry* **18**, 8805–8812.
- Tuckey, R.C. and Holland, J.W. (1989) Comparison of pregnenolone synthesis by cytochrome P-450<sub>scc</sub> in mitochondria from porcine corpora lutea and granulosa cells of follicles. *J. Biol. Chem.* **264**, 5704–5709.
- Tuckey, R.C. and Sadleir, J. (1999) The concentration of adrenodoxin reductase limits cytochrome P450<sub>scc</sub> activity in human placenta. *Eur. J. Biochem.* **263**, 319–325.
- Tusie-Lunam, M.T., Traktman, P. and White, P.C. (1990) Determination of functional effects of mutations in the steroid 21-hydroxylase gene (CYP21) using recombinant vaccinia virus. *J. Biol. Chem.* **265**, 20916–20922.
- Van Rensberg, S.J. (1970) Reproductive physiology and endocrinology of normal and habitually aborting angora goats. D.V.Sc. Thesis, Univ. Pretoria.
- Van Rensberg, S.J. (1973) Reproductive physiology and endocrinology of normal and habitually aborting angora goats. *Onderstepoort J. Vet. Res.* **38**, 1–62.
- Van Voorhis, B.J., Dunn, M.S., Snyder, G.D. and Weiner, C.P. (1994) Nitric oxide: An autocrine regulator of human granulosa-luteal cell steroidogenesis. *Endocrinology* **135**, 1799–1806.
- Viard, I., Rainey, W.E., Capponi, A.M., Begeot, M. and Saez, J.M. (1990) Ovine adrenal fasciculata cells contain angiotensin II receptors coupled to intracellular effectors but are resistant to the steroidogenic effects of this hormone. *Endocrinology* **127**, 2071–2078.
- Voss, A.K. and Fortune, J.E. (1993a) Levels of messenger ribonucleic acid for cholesterol side-chain cleavage cytochrome P-450 and 3 $\beta$ -hydroxysteroid dehydrogenase in bovine preovulatory follicles decrease after the luteinizing hormone surge. *Endocrinology* **132**, 888–894.
- Voss, A.K. and Fortune, J.E. (1993b) Levels of messenger ribonucleic acid for cytochrome P-450 17 $\alpha$ -hydroxylase and P450 aromatase in bovine preovulatory follicles decrease after the luteinizing hormone surge. *Endocrinology* **132**, 2239–2245.
- Voutilainen, R., Tapanainen, J., Chung, B.C., Matteson, K.J. and Miller, W.L. (1986) Hormonal regulation of P450<sub>scc</sub> (20, 22-desmolase) and P450c17 (17 $\alpha$ -hydroxylase/17,20-lyase) in cultured human granulosa cells. *J. Clin. Endocrinol. Metab.* **63**, 202–207.
- White, P.C. (1994) Genetic diseases of steroid metabolism. *Vit. Horm.* **49**, 131–195.



- Wintour, E.M., Crawford, R., McFarlane, A., Moritz, K. and Tangelakis, K. (1995) Regulation and function of the fetal adrenal gland in sheep. *Endocr. Res.* **21**, 81–89.
- Witchel, S.F. and Lee, P.A. (1998) Identification of heterozygotic carriers of 21-hydroxylase deficiency: Sensitivity of ACTH stimulation. *Am. J. Med. Genet.* **76**, 337–342.
- Xu, Z., Garverick, H.A., Smith, G.W., Smith, M.F., Hamilton, S.A. and Youngquist, R.S. (1995) Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome P450 17 $\alpha$ -hydroxylase, and cytochrome P450 aromatase in bovine follicles during the first follicular wave. *Endocrinology* **136**, 981–989.
- Yang, X., Iwamoto, K., Wang, M., Artwohl, J., Mason, J.I. and Pang, S. (1993) Inherited congenital adrenal hyperplasia in the rabbit is caused by a deletion in the gene encoding cytochrome P450 cholesterol side-chain cleavage enzyme. *Endocrinology* **132**, 1977–1982.
- Zuber, M.X., Simpson, E.R. and Waterman, M.R. (1986) Expression of bovine 17 $\alpha$ -hydroxylase cytochrome P-450 cDNA in non-steroidogenic (COS 1) cells. *Science* **234**, 1258–1261.

## 2.

# STEROL BIOSYNTHESIS

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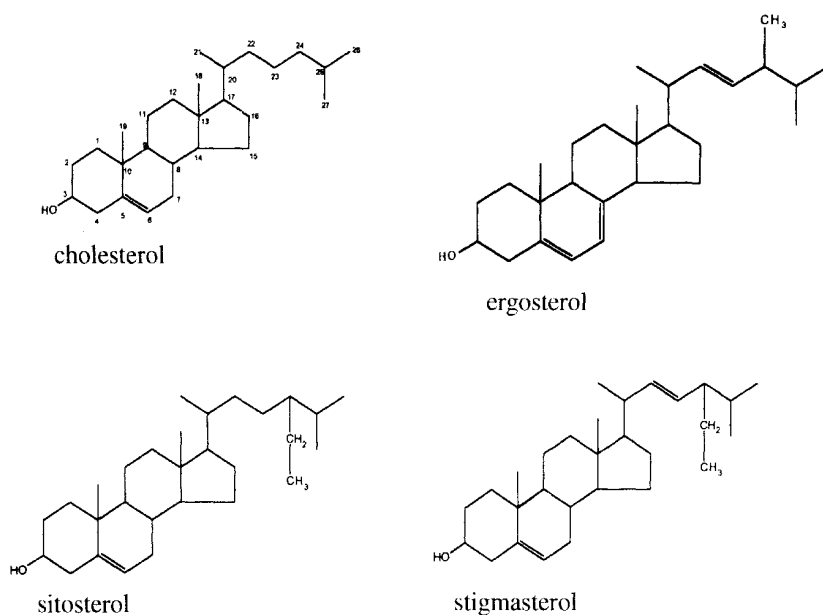
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Sterol biosynthesis is both an elegant and complex biosynthetic process leading to essential lipids in animals, fungi and yeast, plants and perhaps even in some bacteria. While this chapter focuses on synthesis of cholesterol in animals, the biosynthetic pathway is quite conserved in these different phyla. In addition to producing cholesterol, key intermediates in this pathway are utilized in other important biological processes including synthesis of other lipids, farnesylation of proteins and generation of signaling molecules. Regulation of the levels of enzymes in this pathway is controlled by a unique transcription factor, sterol regulatory element binding protein (SREBP), which begins its life as a protein in the endoplasmic reticulum. The transactivation domain of SREBP is proteolytically cleaved from the membrane only under conditions of low sterol concentration, where it enters the nucleus and activates transcription of cholesterologenic genes. Most of these genes in humans are now characterized and their association with genetic diseases and cholesterol biosynthesis is being revealed.

KEY WORDS: cholesterol biosynthesis, SREBP regulation, squalene pathway, postsqualene pathway, MAS sterols.

## INTRODUCTION

Sterols are essential lipids in many but not all phyla. In animals they play many important roles, both species-specific, such as in the formation of the song center in the brain of male birds, and more general, such as reproduction and maintenance of salt balance. In mammals sterols are required not only for steroid hormone biosynthesis, but also for bile acid biosynthesis, vitamin D synthesis and *sonic hedgehog* activity. In addition, sterols in animals, fungi and plants are required for maintenance of membrane fluidity. Most recently the capacity to synthesize sterols has been found in certain bacteria (mycobacteria) but their role in these organisms remains to be determined (Lamb *et al.*, 1998; Bellamine *et al.*, 1999). However, most bacteria do not make sterols. Figure 2.1 shows sterols produced in animals (cholesterol), fungi (ergosterol) and plants (phytosterols). In *Mycobacterium smegmatis* cholesterol can be produced from mevalonate, so presumably cholesterol is the sterol produced in some bacteria (Lamb *et al.*, 1998). The genome of *Caenorhabditis elegans* does



**Figure 2.1 Major sterol products in animals (cholesterol), fungi and yeast (ergosterol) and plants (sitosterol and stigmasterol)**

not contain genes encoding a sterol biosynthetic pathway (Ainscough *et al.*, 1998), nor does the genome of *Drosophila melanogaster* (Adams *et al.*, 2000). If sterols are important for nematodes and insects, they must be obtained from the environment. The mechanisms by which sterols are synthesized, transported throughout organisms and metabolized are very well studied. This chapter provides an overview of the biosynthesis of sterols, being focused on mammalian sterol (cholesterol) biosynthesis. It will become apparent that this process not only leads to essential sterols, but that intermediates in sterol biosynthesis are used for multiple metabolic purposes, and that several enzymes of the pathway seem to have additional, receptorlike properties. Thus, the importance of sterol biosynthesis is complex and profound.

In developed societies, cholesterol is often viewed as man's enemy because of its connection to coronary artery disease. However, it must not be forgotten that cholesterol is an essential component of cell membranes, that it is required for cell division and that the absence of cholesterol in embryonal stages of development leads to severe malformations or is even lethal. Although as much as 20% of fetal cholesterol may be derived from the mother in the first trimester of pregnancy, very little cholesterol enters the fetal brain, development of which depends essentially on *de novo* cholesterol synthesis (Clayton, 1998). Daily,

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approximately 1000 mg of cholesterol are introduced into the body from the diet (data for North American population; Salen *et al.*, 1996a), of which 300–500 mg are absorbed into the alimentary tract. Still more cholesterol is synthesized in cells *de novo*; daily at least 1000 mg of newly synthesized cholesterol is necessary (Salen *et al.*, 1996a). While all nucleated cells can synthesize cholesterol, 70% of this lipid is produced in the liver. Especially rich in cholesterol are myelin structures of the brain and the central nervous system. Cholesterol and its biologically active products should be present in cells at the right time and in correct concentrations. This is guaranteed by precise and co-ordinated control of the uptake cholesterol from the diet and the *de novo* synthesis of cholesterol, through sterol-dependent transcription factors of the family SREBP (Goldstein and Brown, 1990).

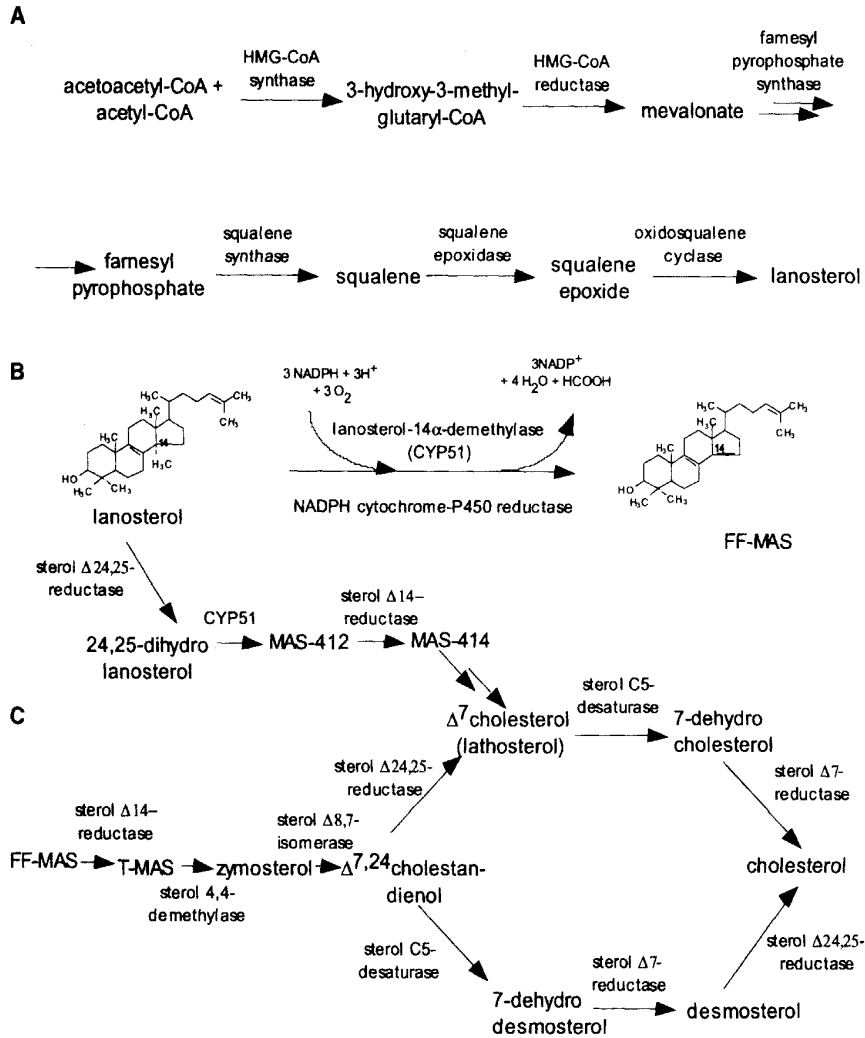
Cholesterol itself is an active molecule binding and activating the transcription factor *sonic hedgehog*, one of the housekeeping genes expressed during embryogenesis. Animals with a disrupted *sonic hedgehog* gene (Chiang *et al.*, 1996; Porter *et al.*, 1996) exhibit holoprosencephalitis, the same phenotype as rodents exposed to inhibitors of the post-squalene part of cholesterol biosynthesis, AY9944 and BM15766 (Honda *et al.*, 1996; Wolf *et al.*, 1996). Also patients with the Smith-Lemli-Opitz syndrome (SLOS) exhibit a similar phenotype. Adding cholesterol into the food of patients, suffering from defects in its biosynthesis, can improve the clinical symptoms. However, because of the capacity of humans to synthesize cholesterol, for healthy people of all ages the World Health Organization recommends “the less—the better” cholesterol in the diet, since there is no firm proof that lack of dietary cholesterol can influence normal development (Barness, 1994).

## ENZYMES OF THE SQUALENE PORTION OF CHOLESTEROL BIOSYNTHESIS

Cholesterol contains 27 carbon atoms, all of which arise from acetate. The biosynthetic pathway involves at least 17 enzymes and is one of the most remarkable chemical syntheses found in biology (Figure 2.2). In a broad sense the biosynthetic pathway can be divided into two parts: the squalene portion and the post-squalene portion. In the squalene portion, C2 acetate units are converted to C6 isoprenoid units, which are then condensed to form the C30 squalene molecule. In the post-squalene portion of the biosynthetic pathway, squalene is cyclized and the 30 carbon intermediate lanosterol is transformed to the 27 carbon cholesterol molecule. The capacity to synthesize cholesterol is found in all cells with the liver being the major factory. The synthesis of squalene has been studied in great detail and is well understood and described in most biochemistry text books. Therefore it will be described only briefly here with the major emphasis of this chapter being on the post-squalene portion of the cholesterol biosynthetic pathway.

### HMGCoA biosynthesis

The first steps in cholesterol biosynthesis take place in the cytosol where two molecules of acetyl-CoA are converted to acetoacetyl-CoA by the cytosolic isoform of  $\beta$ -ketothiolase. 3-



**Figure 2.2 Overview of the cholesterol biosynthetic pathway.** (A) Synthesis of lanosterol, (B) Synthesis of FF-MAS from lanosterol, (C) Conversion of MAS sterols to cholesterol.

Hydroxy-3-methylglutaryl-CoA (HMG-CoA) is formed from acetoacetyl-CoA and acetyl-CoA by HMG-CoA synthase.

**HMG-Co reductase and mevalonate formation**

HMG-Co reductase is the key enzyme in the complete biosynthetic pathway and is located in the endoplasmic reticulum (ER). It catalyzes NADPH-dependent reduction of HMG-CoA to

mevalonate which is the first committed step in the cholesterol biosynthetic pathway and is the most highly regulated, involving at least three different regulatory mechanisms. The enzyme is inactivated by phosphorylation via a cAMP-dependent protein kinase, which can be reversed by phosphatases. HMG-CoA reductase has a short half-life (~3 hours) and higher cholesterol levels even shorten this half-life. A third regulatory mechanism involves transcription via the transcription factor SREBP. Details of the function of SREBP are described later in this chapter since it probably regulates transcription of all cholesterologenic genes.

### **Conversion of mevalonate to squalene**

The next several steps of squalene biosynthesis take place in the cytosol. Mevalonate kinase catalyzes two ATP-dependent steps forming pyrophosphomevalonate. Phosphomevalonate decarboxylase then phosphorylates the 3-hydroxyl group and catalyzes decarboxylation via trans-elimination to produce isopentyl pyrophosphate. Isopentyl pyrophosphate can isomerize to dimethylallyl pyrophosphate via isopentyl pyrophosphate isomerase. Isopentyl pyrophosphate and dimethylallyl pyrophosphate condense to form the 10 carbon geranyl pyrophosphate. Geranyl pyrophosphate condenses with a second molecule of isopentyl pyrophosphate to produce the 15 carbon farnesyl pyrophosphate. Both condensations are catalyzed by prenyl transferase. The next enzyme, squalene synthase, is located in the ER and joins two molecules of farnesyl pyrophosphate producing the 30 carbon squalene in a reaction that requires NADPH.

Certain intermediates in the conversion of mevalonate to squalene play key roles in biological processes in addition to cholesterol biosynthesis. Isopentyl pyrophosphate is the precursor for a large number of molecules in animals and plants. These include vitamins A, E and K and ubiquinone (coenzyme Q) in animals and sesquiterpenes, plastoquinone and the phytyl side chain of chlorophyll in plants. Farnesyl pyrophosphate is a key intermediate in synthesis of the lipid dolichol and in farnesylation of proteins where the polyprenyl group serves as a membrane anchor.

## **ENZYMES OF THE POST-SQUALENE PORTION OF CHOLESTEROL BIOSYNTHESIS**

### **Cyclization of squalene**

Squalene monooxygenase (previously known as squalene epoxidase) is a FAD containing enzyme located in the ER. It catalyzes the epoxidation of squalene producing 2,3-oxidosqualene in a reaction requiring NADPH. This protein has been cloned from both rats (Sakakibara *et al.*, 1995) and humans (Laden *et al.*, 2000). Another ER enzyme, oxidosqualene cyclase, converts 2,3-oxidosqualene to lanosterol.

### **Lanosterol 14 $\alpha$ -demethylase (CYP51), a member of the cytochrome P450 superfamily**

Lanosterol 14 $\alpha$ -demethylase belongs to the superfamily of cytochromes P450. In mammals, this superfamily includes enzymes involved in the endogenous metabolism (synthesis of steroid hormones, of bile acids, prostaglandins, vitamin D, etc.) as well as families of enzymes responsible for detoxification of exogenous substances (xenobiotics). CYP51 resides in the ER being embedded into the membrane by an N-terminal signal anchor sequence. Like all P450s in the ER, it requires electron transfer from NADPH P450 reductase for its activity. CYP51 removes the methyl group at position 14 $\alpha$  from the substrates lanosterol and 24,25-dihydrolanosterol, leading to 4, 4-dimethyl-5 $\alpha$ -cholesta-8, 14,24-triene-3 $\beta$ -ol, also called FF-MAS (follicular fluid meiosis activating sterol) or to its 23,25-dihydro analogue MAS-412 (Figure 2.2). Recent studies (Byskov *et al.*, 1995) have indicated that the direct products of CYP51 and the next enzyme in the biosynthetic pathway, sterol  $\Delta^{14}$ -reductase (sterols FF-MAS and T-MAS, respectively) accumulate in ovaries and testes. It has been shown that these sterols isolated from tissues (Byskov *et al.*, 1995) as well as their chemically synthesized forms (Grondahl *et al.*, 1998) can reactivate meiosis in mouse oocytes *in vitro*, hence their name. The role of MAS sterols *in vivo* has not yet been clarified. CYP51 mRNA is ubiquitously expressed showing expression levels in testis one order of magnitude higher than in other tissues (Strömstedt *et al.*, 1998). CYP51 mRNA as well as the protein (also known as P45014DM) are found at maximal levels in spermatides, male germ cells which have already completed meiosis (Majdič *et al.*, 2000), suggesting that the role of MAS sterols during spermatogenesis (in contrast to oogenesis) may not be in meiosis. A specific receptor which would bind lipophilic MAS sterols enabling its transfer through the blood or cytoplasm has not yet been discovered. Recent data indicate that MAS sterols do not influence transcription suggesting that rather than nuclear receptors, the MAS receptor might be linked to a G-protein coupled mechanism (Grondahl *et al.*, 2000).

The fact that CYP51 mRNA is an order of magnitude higher in testis than liver is surprising since liver is responsible for over 70% of the body's cholesterol biosynthesis. Testis is also the organ where highest levels of MAS sterols are observed (Baltsen and Byskov, 1999). CYP51 mRNA is expressed in a stage-specific manner during spermatogenesis of the rat, mouse and human, with highest mRNA levels found in round and elongated spermatides (Rozman and Waterman, 1998; Strömstedt *et al.*, 1998; Rozman, 2000). The high levels of CYP51 mRNA therein is due to the appearance of 3' shortened, germ cell-specific transcripts, which arise by using more upstream polyadenylation sites as compared to CYP51 mRNAs in other cell types (somatic cells). A cAMP-dependent mechanism of CYP51 transcriptional activation in postmeiotic germ cells is mediated by the cAMP-responsive element modulator CREM. This type of regulation differs from the sterol/SREBP-dependent transcriptional activation in the liver and other somatic cells (Rozman *et al.*, 1999). The CREMt-dependent upregulation of CYP51 is the first example of tissue-specific, sterol-independent regulation of a cholesterologenic gene. Other genes of the postsqualene part of cholesterol biosynthesis are not regulated by a CREMt-dependent mechanism (Fon-Tacer *et al.*, 2002). Thus, the biosynthetic pathway seems to have no co-ordinate regulation in male germ cells, while a co-ordinate, SREBP-dependent upregulation exists in the liver and other somatic cells (Brown and Goldstein,

1998). We propose that the discordant regulation in germ cells cannot lead to increased amounts of cholesterol, but rather to increased levels of pathway intermediates, such as FF-MAS and T-MAS.

The CYP51 cDNA has been cloned from rat (Aoyama *et al.*, 1994; Sloane *et al.*, 1995; Noshiro *et al.*, 1997), human (Strömstedt *et al.*, 1996) and mouse (Debeljak *et al.*, 2000). Three CYP51-like genes exist in the human genome, a functional gene on chromosome 7q21.2–21.3 (Rozman *et al.*, 1996a), and two intronless, processed pseudogenes on chromosomes 3 and 13 (Rozman *et al.*, 1996b). No genetic disease connected to mutations in the CYP51 gene is known as it is believed that the enzyme is essential in the embryonal development because the sterols with a 14-methyl group cannot efficiently substitute for membrane cholesterol. However, a human cell line A3.01 isolated from a patient with acute cell leukemia, has no detectable cholesterol whereas the 14-methyl sterols, 24,25-dihydrolanosterol and lanosterol, represent respectively 75% and 25% of cell sterols (Buttke and Folks, 1992).

### **Is sterol $\Delta^{14}$ -reductase a member of the lamin B receptor/ sterol reductase multigene family?**

Sterol  $\Delta^{14}$ -reductase, which transforms FF-MAS into T-MAS, has been reported to belong to the lamin B receptor/sterol reductase multigene family (Holmer *et al.*, 1998). Lamin B receptor is an integral protein of the inner nuclear membrane. It is composed of two parts, a basic N-terminal domain facing the nucleoplasm and a C-terminal portion which contains eight transmembrane segments showing striking homology to yeast and plant sterol reductases. A phylogenetic tree shows that the yeast and plant sterol reductases ( $\Delta^{14}$ ,  $\Delta^7$  and  $\Delta^{24,25}$ ) root together with the human lamin B receptor (Holmer *et al.*, 1998). However, over-expression of the chicken lamin B receptor does not complement the sterol  $\Delta^{14}$ -reductase deficiency in *S. cerevisiae* (Lecain *et al.*, 1996), showing that it does not harbor the reductase activity. The human lamin B receptor gene resides on chromosome 1q42.1. Although the C-terminal domain of this receptor can exhibit sterol  $\Delta^{14}$ -reductase activity (Silve *et al.*, 1998) considerable controversy remains as to which protein is the true sterol  $\Delta^{14}$ -reductase. While the lamin B receptor is normally located in the nucleus, two related proteins have been detected in the ER. The genes encoding both proteins map to the human chromosome 11q13 and one of these two genes could also be the sterol  $\Delta^{14}$ -reductase (Holmer *et al.*, 1998). In earlier studies, the rat sterol  $\Delta^{14}$ -reductase was identified as a microsomal 70 kDa homodimer which is able to catalyze a NADPH-dependent C14 double bond reduction of  $\Delta^{8,14}$ - or  $\Delta^{8,7,14}$ -sterols. Its activity is inducible by HMG-CoA reductase inhibitors (e.g. lovastatin) and cholestyramine (a bile salt sequestrator) and is inhibited by cholesterol and AY9944 (Kim *et al.*, 1995). However, identification of the gene encoding the protein which transforms FF-MAS into T-MAS *in vivo* awaits determination.



### **Sterol 4,4-demethylase complex, belonging to the membrane desaturase/hydroxylase family**

Early data show that sterol 4,4-demethylase does not belong to the cytochrome P450 family. It was insensitive to inhibition by CO but was rather a cyanide-sensitive enzyme (Taton *et al.*, 1994). It catalyzed removal of the methyl groups at position 4 from T-MAS by a mixed function oxidase reaction (Gaylor and Delwiche, 1976). The levels of this enzyme were inhibited by cholesterol and its oxygenated metabolites. While data on the structure of the gene encoding this enzyme in mammals are circumstantial, sterol 4,4-demethylases are well characterized in yeasts and plants. A human cDNA clone having 38% amino acid identity to yeast sterol 4,4-demethylase has been identified, mapping to the chromosome 4q32–34 (Liangtao and Kaplan, 1996). The hydropathy analysis of the deduced human protein shows four potential transmembrane domains, as is characteristic for the yeast and plant proteins. It seems that sterol C5-desaturase, another enzyme involved in the latter steps of cholesterologenesis, is phylogenetically close to sterol 4,4-demethylase (Liangtao and Kaplan, 1996). While in yeasts both methyl groups in position 4 of the steroid skeleton are removed in a single enzymatic step, two steps are needed to complete this reaction in plants (Taton *et al.*, 1994) and at least two enzymatic steps are needed in mammals (Liu *et al.*, 1999). Deficiency of sterol 4,4-demethylase activity in humans is linked to the genetic disease chondrodysplasia punctata (CDPXZ) (Liu *et al.*, 1999) and to mouse mutations bare patches (Bp) and striated (Str).

### **Sterol $\Delta^{8,7}$ -isomerase, a member of the sigma receptor family**

Sterol  $\Delta^{8,7}$ -isomerase catalyzes a displacement of a double bond from position 7 to position 8 in substrates zymosterol and 24,25-dihydrozymosterol. Due to recently discovered association with the sigma receptor family, intensive research has been directed to the development of isomerase inhibitors. Sigma receptors bind drugs and appear in ER membranes in brain and endocrine tissues. The proposed transmembrane topology of the sigma 1 receptor, based on the hydrophobicity plot, is identical to yeast and plant sterol  $\Delta^{8,7}$ -isomerases, showing four transmembrane domains (Moebius *et al.*, 1997). The receptor is responsible for mediating various pharmacological effects (immunosuppressive, antipsychotic and neuroprotective) of sigma ligands, such as haloperidol, pentazocyn, etc., by as yet unknown mechanisms. Because endogenous ligands for sigma receptors are not known, these receptors were defined as proteins, which bind high affinity haloperoid-sensitive drugs. One of the two cloned human genes exhibits a 30% amino acid homology with the yeast sterol  $\Delta^{8,7}$ -isomerase, however, it is functionally unable to substitute the yeast isomerase in ergosterol biosynthesis (Jbilo *et al.*, 1997). The other cloned gene, characterized as the emopamil binding protein, exhibits only a 17% homology with the sterol isomerase of yeasts, yet can complement the loss of this enzyme in yeast (Silve *et al.*, 1996). Although there is no doubt that two of the three representatives of the sigma receptor family can act as sterol isomerases, we do not know as yet which play (s) the role of sterol isomerase in different mammalian tissues. Some believe that sigma receptors are sterol  $\Delta^{8,7}$ -isomerases characteristic for the brain, however, the picture may be more complex. Because sigma ligands have anti-ischemic effects on animal models of stroke, it can be presumed that

inhibitors of cholesterol biosynthesis may influence the proliferation of nervous cells preventing cell death (Moebius *et al.*, 1997, 1998b). Interesting, human genetic disease associated with this gene is also chondrodysplasia punctata (CDPX2), as in the case of 4,4-demethylase deficiency. However, a different mouse mutation, tattered (Td), is associated with the disease (Derry *et al.*, 1999).

### **Sterol C5-desaturase, a member of the membrane desaturase/hydroxylase family?**

Sterol C5-desaturase catalyzes the formation of the C5-double bond in the B ring of cholesterol by a mixed-function oxidase reaction, requiring a multienzyme electron transport system for its activity (Grinstead and Gaylor, 1982; Kawata *et al.*, 1985). It catalyzes the cis-desaturation of  $\Delta^7$ -sterols to form 7-dehydrocholesterol. The mammalian gene is a homologue of the yeast *erg3* gene. The human gene lies on chromosome 11q23.3 and is ubiquitously expressed (Matsushima *et al.*, 1996). There is little information about its precise physiological role and regulation and mutations in this gene are not known.

### **Sterol $\Delta^7$ -reductase, a member of the lamin B receptor/sterol reductase multigene family**

Reduction of the  $\Delta^7$ -double bond of sterols is a key biosynthetic step in higher eukaryotes, while it is absent in lower eukaryotes (Lecain *et al.*, 1996). The sterol  $\Delta^7$ -reductase gene has been cloned first in *A. thaliana* and shown to belong to the lamin B receptor family, together with the yeast sterol  $\Delta^{14}$ - and  $\Delta^{24,(28)}$ -reductases. Sterol  $\Delta^7$ -reductase has long been considered the primary candidate for an autosomal recessive disease, the Smith-Lemli-Opitz syndrome. The suspicion was shown to be correct recently when the corresponding human gene was cloned (Moebius *et al.*, 1998a). The human gene maps to chromosome 11q13 and the mouse homolog to the 7F5 (Fitzky *et al.*, 1998). Sterol  $\Delta^7$ -reductase is a nine transmembrane domain protein. It is ubiquitously expressed in humans with the highest level in the adrenal glands, liver, testis and the brain.

### **Sterol $\Delta^{24,25}$ -reductase, a member of the lamin B receptor/sterol reductase superfamily**

Sterol  $\Delta^{24,25}$ -reductase catalyzes the reduction of the side chain of a series of obligatory intermediates of cholesterol biosynthesis derived from lanosterol. Consequently, two parallel paths of cholesterol biosynthesis exist, either through 24,25-oxygenated or 24,25-reduced intermediates. Although the precise location of the branching  $\Delta^{24,25}$ -reductase step is not known, since the enzyme can provide C-24 reduction on all sterol intermediates containing the D24,25-double bond,  $\Delta^{7,24}$ -cholestan-dienol (5 $\alpha$ -cholesta-7,24-diene-3 $\beta$ -ol) seems to be the best substrate (Bae and Paik, 1997). Figure 2C indicates these two routes and the intermediate sterols isolated from tissues. The branching reaction pathway from lanosterol (Figure 2.2B, C) is markedly increased under conditions of sterol deprivation (Bae and Paik, 1997). The  $\Delta^{24,25}$ -reductase activity is induced as much as 120-fold by

inhibitors of cholesterol biosynthesis, and under such conditions the preferred substrate is lanosterol. A particular branch path may be tissue-specific, yet this issue has not been well studied. In view of the structural similarities of sterol reductase of yeast and mammals with lamin B receptors, it is presumed that the sterol  $\Delta^{24,25}$ -reductase is also a member of this receptor family. The gene for this enzyme, which has several trivial names (lanosterol reductase, desmosterol reductase), has initially been localized to human chromosome 20. However, studies of patients with desmosterolosis that is carried by mutations in the sterol  $\Delta^{24,25}$ -reductase gene have localized the DHCR24 gene to human chromosome 1p31.1-p33 (Waterman *et al.*, 2001).

### COORDINATE CONTROL OF BIOSYNTHESIS OF CHOLESTEROL WITH TRANSCRIPTION FACTORS OF THE SREBP FAMILY

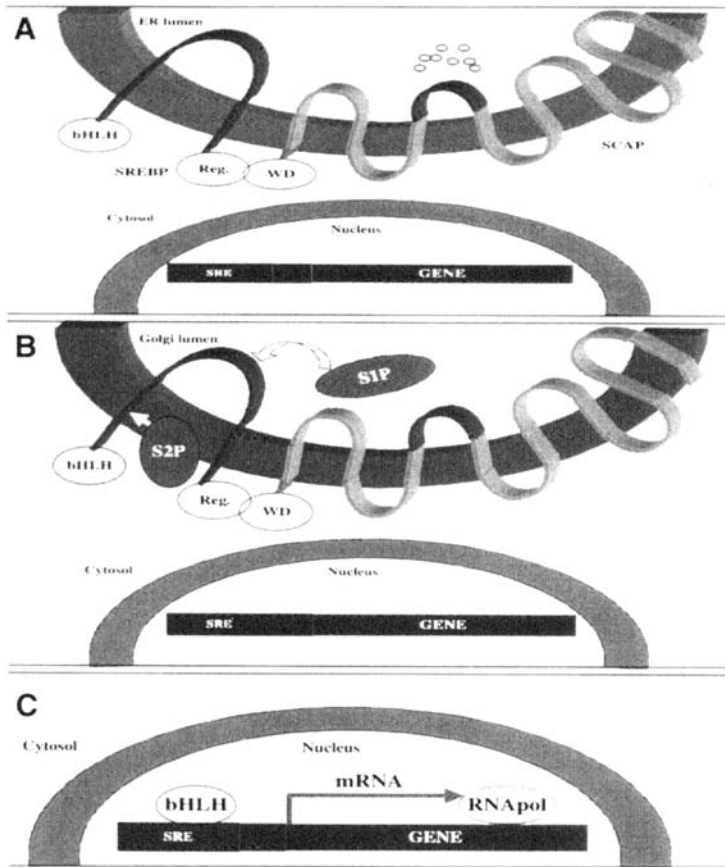
Starvation of cholesterol, an essential component of cellular membranes, is one of the elicitors of alteration in endoplasmic reticulum (ER) structure (ER stress) (Pahl, 1999). Until the early 1990s very little was known about how cells respond to ER stress, which transcription factors are involved and by what mechanisms they are induced. It is now well established that the sterol regulatory element binding protein (SREBP) pathway regulates cholesterol synthesis in response to lowered sterol plasma levels (Pahl, 1999). Regulation is mediated at the level of transcription, by inducing mRNA levels of sterol-dependent genes, leading to increased amounts of the corresponding cholesterologenic enzymes. Synthesis of all enzymes of cholesterol biosynthesis, as well as the synthesis of the LDL receptor, which is responsible for uptake of LDL-bound cholesterol acquired in the diet are controlled in a coordinated manner by SREBPs. In addition to cholesterol biosynthesis and uptake, these transcription factors also regulate fatty acid synthesis and uptake. It is now believed that SREBPs regulate membrane synthesis, and that the integrity of cell membranes is maintained by a SREBP-dependent balance between the amount of cholesterol and the amounts of unsaturated and saturated fatty acids, as well as phospholipids (Brown and Goldstein, 1999).

SREBP proteins belong to a group of basic helix-loop-helix-leucine-zipper (bHLH-Zip) proteins. They are membrane-bound, composed of three domains (~1150 amino acids), and reside in the nuclear envelope and ER membranes in a hairpin orientation. The N-terminal domain (~480 amino acids) and the C-terminal domain (~590 amino acids) project into the cytosol. Only the N-terminal domain containing the  $\beta$ HLLH-Zip DNA binding motif is transcriptionally active (Brown and Goldstein, 1999). Decline of cholesterol content in a cell is a signal for upregulation of its biosynthesis (Brown and Goldstein, 1997). A sterol-sensitive proteinase (site-1 protease, S1P, a membrane-bound serine proteinase of the subtilisin family) cleaves the hydrophilic loop of SREBP that projects into the ER lumen. The second cleavage is performed by a sterol-insensitive proteinase (Site-2 proteinase, S2P, a zinc metalloproteinase) within the membrane-spanning domain of SREBP (Sakai *et al.*, 1996; Brown and Goldstein, 1999). Thus the N-terminal, transactivating  $\beta$ HLLH-Zip domain of the SREBP protein is released, enters the nucleus and binds to SRE regulatory elements of responsive genes. SREBPs were the first studied example of membrane-bound transcription factors, which are now known to belong to a group of proteins activated by the

regulated intramembrane proteolysis (Rip) (Brown *et al.*, 2000). Sterols (cholesterol and oxygenated derivatives) block SREBP by preventing its availability to the site-1 proteinase, leaving sterol-responsive genes in a repressed state (Figure 2.3A). Another membrane bound protein, the SREBP activating cleavage protein (SCAP), serves as a sterol sensor. SCAP is responsible for the transport of its substrate SREBP from the ER to the post-ER compartment (*cis* or medial Golgi), where activated SIP resides (Brown *et al.*, 2000). Mutant cell lines lacking SCAP are unable to cleave SREBPs because the membrane-bound SREBP precursor is not transported to the proper cellular compartment (Figure 2.3B). When normal cells are deprived of cholesterol, the membrane-bound SCAP/SREBP complex, both proteins interacting through their C-terminal domains, moves to the cleavage compartment. Conversely, when cells have sufficient sterols the SCAP/SREBP complex remains trapped in the ER, preventing cleavage (Brown *et al.*, 2000).

Upon activation, the  $\beta$ HLH-Zip domain of SREBP transcription factors bind to the SRE regulatory elements in promoter regions of sterol-responsive genes, upregulating their transcription (Figure 2.3C). The  $\beta$ HLH-Zip motif is responsible for dimerization of the transcription factor and for nuclear entry as well as for DNA binding. SREBPs do not recognize classical palindromic E-box elements as characteristic for other  $\beta$ HLH-Zip transcription factors. An amino acid change in the basic region of the DNA-binding domain (Arg→Tyr) allows SREBP to recognize decanucleotide DNA segments, called sterol regulatory elements (SREs) (Brown and Goldstein, 1999). Although the main regulatory enzyme of cholesterol biosynthesis is HMG-CoA reductase, 5'-nontranscribed regions of all known cholesterologenic genes contain SREs (Rozman *et al.*, 1999). The sequence of SRE elements differ in various genes, the originally defined sequence being ATCACCCAC found in the LDL-receptor and HMG-CoA synthase genes (Athaniar and Osborne, 1998). It is believed that controlling each gene individually enables a precise regulation, fine tuning, in each step of this metabolic path. There are three forms of transcription factor SREBP: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1a and 1c are encoded by the same gene and arise by differential splicing, resulting in a different sequence of the first exon leading to two proteins having different N-terminal domains (Yokoyama *et al.*, 1993). SREBP-1a is a stronger activator of transcription than SREBP-1c (Shimano *et al.*, 1997a) and different cell types can contain different quantities of either protein (Shimomura *et al.*, 1997).

Transgenic mouse models have been developed constitutively over-expressing the transactivating, nuclear forms of SREBP-1a (Shimano *et al.*, 1996) or SREBP2 in mice livers (Horton *et al.*, 1998). Because the nuclear form of the transcription factor SREBP is present, the mechanism of control by oxysterols is eliminated. In this manner, genes in wild type animals that are induced by a lower content of cholesterol are constitutively over-expressed in livers of SREBP-transgenic animals. It is not known yet whether any of the hypercholesterolemia forms in humans correspond to the phenotype of SREBP over-expression in transgenic mice. Besides SREBP transgenic animals, the SREBP knockout models have also been developed. It is interesting that disruption of the transcription factor SREBP-1 is not necessarily lethal as 15–50% of SREBP-1<sup>-/-</sup> mice develop to adulthood (Shimano *et al.*, 1997b). These animals exhibit an increased expression of the transcription factor SREBP-2, which shows that SREBP-2 can partly substitute a lack of SREBP-1. On the contrary, disruption of the SREBP-2 gene is lethal early in the embryonal stage (Shimano *et*



**Figure 2.3 Overview of action of sterols in regulating SREBP-dependent transcription in cholesterol synthesizing cells with permission from M.Cotman, MSc thesis, University of Ljubljana, 2000.** (A) Repression of SREBP cleavage under conditions of high sterol levels. Circles represent sterols (cholesterol or oxidized derivatives). SREBP and SCAP are defined in the text.  $\beta$ HLH designates the basic helix-loop-helix DNA binding domain at the N-terminus of SREBP. Reg refers to the regulatory region of SREBP (C-terminus) which does not interact with the C-terminus (WD) of SCAP under these conditions. (B) When the sterol concentration is lowered the C-termini of SREBP and SCAP interact and SCAP transports SREBP to the Golgi where activated sterol protease 1 (S1P) cleaves SREBP in the loop located in the lumen. Sterol protease 2 (S2P) then cleaves SREBP within the membrane, releasing the N-terminal  $\beta$ HLH transactivating domain. (C) This transactivation domain of SREBP then enters the nucleus and binds to SRE elements in the promoter region of cholesterologenic genes, enhancing their transcription by RNA polymerase II (RNA pol.).

*al.*, 1997b), which shows that SREBP-1 cannot complement SREBP-2 in embryonic development. *In vivo*, SREBP-2 is the main activator of sterol-dependent activation of genes

involved in cholesterol biosynthesis while SREBP-1a more efficiently activates fatty acid synthesis. No genetic diseases connected to mutations in the SREBP genes are known. The ability to control cholesterol biosynthesis is of decisive importance for a normal development of an embryo.

## GENETIC DISEASES OF THE POST-SQUALENE PATHWAY

The number of genetic diseases connected to the post squalene portion of cholesterol formation is increasing. This part focuses only on the two best characterized diseases (SLOS and desmosterolosis for which single gene mutations are linked to the disease phenotype).

### Smith-Lemli-Opitz syndrome (SLOS)

SLOS syndrome is an inborn disorder of sterol metabolism with characteristic congenital malformations and dysmorphisms. It is caused by mutations in the sterol  $\Delta^7$ -reductase gene and is the best studied example of a syndrome where blocking of a single metabolic path causes a series of different damages of the organism (Kelley *et al.*, 1996). The disease was described in 1964 by David Smith, Luc Lemli and John Opitz (Smith *et al.*, 1964), and chemical tests enabling diagnosis of the syndrome have recently been developed (Irons *et al.*, 1993, 1994; Tint *et al.*, 1994). The SLOS patients are mentally retarded. According to the phenotype, the disease is expressed as a milder type (Type I) or a more serious type (Type II) with heterogeneous symptoms, such as microencephalitis, flat feet, cleft palate, syndactylia of the index and middle fingers, polydactyly, and visceral malformations. The genitals of seriously affected boys remain phenotypically female. Patients inflicted with SLOS exhibit a 3–15 times lower quantity of cholesterol than control subjects (143±20 mg/dl). It is presumed that embryos with a concentration of cholesterol below 7mg/dl do not survive (Salen *et al.*, 1996b). Phenotype changes of the afflicted confirm that biosynthesis of cholesterol is essential in the embryonic development. Cholesterol serves not only as a precursor of steroidogenic molecules and membrane components, but also as activator of the signal protein *sonic hedgehog* (*sh*) (Kelley *et al.*, 1996; Lanoue *et al.*, 1997). *Sh* is one of the proteins responsible for a normal segmentation of the embryo in early development, lack of cholesterol meaning lack of active form of protein *sh*. Besides the lack of cholesterol, the clinical picture of the disease can also be influenced by abnormally high concentrations of sterols, which are present in healthy individuals in negligible quantities. Concentrations of 7-dehydro- or 8-dehydrocholesterol in SLOS patients may be as high as 20 mg/dl (Salen *et al.*, 1996b), whereas in the plasma higher quantities of 19-nor-5,7,9(10) cholestatrienol can appear (Batta *et al.*, 1995). The occurrence of the syndrome is 1 in 20,000 births, the frequency of the carrier of disease in North American Caucasian population being 1–2%. SLOS syndrome is the third (Salen *et al.*, 1996b), or even the second (Tint *et al.*, 1994) most frequent genetic disease of the North American Caucasian population. In some Eastern European populations the disease is even more frequent. In Czechs, the occurrence is 1 in 9000 babies born alive or born dead, one of every fifty Czechs being a carrier (Opitz, 1994). For a comparison, the occurrence of the three other most frequent meta-bolic diseases observed during childhood is 1 in 800 births for cystic fibrosis, 1 in 14,000 for

phenylketonuria and 1 in 40,000 births for galactosemia. Because cholesterol is vitally necessary as a structural component of all cells, it is presumed that the most serious damages of the gene of sterol  $\Delta^7$ -reductase leads to spontaneous miscarriages in early stages of pregnancy, so that the true occurrence of SLOS syndrome is probably much higher.

There is an increasing number of studies describing mutations in the gene of sterol  $\Delta^7$ -reductase by SLOS patients. In a study including thirteen SLOS patients from seven German centers, different missense and nonsense mutations have been found (Fitzky *et al.*, 1998). American SLOS patients also exhibit different types of mutations, particularly insertions and large deletions (Wassif *et al.*, 1998). A recent study including 84 patients from different countries allowed grouping the SLOS mutations into 4 groups: (a) nonsense and splice site mutations, resulting in putative null alleles; (b) missense mutations in the transmembrane domain (TM); (c) mutations of the 4th cytoplasmic loop of the protein (4L); and (d) mutations in the C-terminal domain associated with the ER (CT) (Witsch-Baumgartner *et al.*, 2000). The mildest clinical phenotypes are associated with CT and TM mutations while the severe phenotypes include null or 4L mutations. It is interesting to note that homozygosity for null mutations in the sterol  $\Delta^7$ -reductase gene seems to be compatible with embryonal survival and life. This suggests that either exogenous sources of cholesterol may be used during development, or that cholesterol may be synthesized also in the absence of this terminal enzyme of the pathway (Witsch-Baumgartner *et al.*, 2000). Because all born SLOS patients have severe malformations, methods for prenatal diagnosis of the disease have been developed and cases are described (Hyett *et al.*, 1995; Mills *et al.*, 1996; Tint *et al.*, 1998). The analysis of sterols of amniotic fluid from a larger number of females who had miscarried affected embryos, showed that measuring concentrations of cholesterol, 7-dehydrocholesterol and 8-dehydrocholesterol is a clinically relevant indication for the SLOS-affected embryo (Tint *et al.*, 1998).

### Desmosterolosis

The elucidation of the biochemical basis of SLOS syndrome has led to a search for accumulation of precursors of cholesterol in tissues from children with similar malformation phenotypes. This search led to discovery of a new genetic disease, desmosterolosis, where a damage in the gene for sterol  $\Delta^{24,25}$ -reductase is expected to be impaired (Clayton, 1998). The nucleotide sequence of this mammalian gene is not known yet, but it is presumed that the gene lies on human chromosome 20 (Clayton *et al.*, 1996). Desmosterolosis was discovered by analysis of sterols in embryos after natural miscarriages, analysis of sterols of babies born dead and of babies born alive, but with phenotypes similar to SLOS syndrome (Clayton *et al.*, 1996; FitzPatrick *et al.*, 1998). In all cases, an abnormal development of the brain and the genitals was observed and additional malformations were observed. In a case of one of the babies born dead, 77% of desmosterol and only 23% of cholesterol was found in the brain while by healthy individuals desmosterol does not exceed 15% of the total of sterols. Greater quantities of desmosterol were also observed in the kidneys and liver. Because the parents of this stillborn baby also had slightly elevated quantities of desmosterol, it was concluded that they were heterozygotes. This example of desmosterolosis suggests the role of normal endogenous cholesterol production for the development of the human brain and

the craniofacial, the heart, the skeleton and the urogenital systems (Clayton *et al.*, 1996). The disease is rare, an extract frequency is not known as many homozygotic pregnancies most probably end with miscarriage. The detailed mutation analysis will be possible only after the gene is characterized (Clayton, 1998).

### CONCLUDING REMARKS

In the last four years, enormous progress in comprehension of genes and enzymatic activities of the postsqualene part of biosynthesis of cholesterol has occurred. It can be expected that in the near future all missing parts of the metabolic path will be genetically characterized. The discovery of the biochemical details of regulation cholesterol biosynthesis by lowered cholesterol levels (SREBP) has been full of surprises and it can be predicted that there are more to come. Furthermore, the association of enzymes in the postsqualene part of cholesterol biosynthesis with gene families having quite different functions than lipid metabolism is sure to provide additional surprises in the near future. The chemical steps in sterol biosynthesis have been long known to be quite a remarkable biological process. It is now becoming increasingly clear that the regulation of this pathway and the enzymes themselves are also quite remarkable. Nothing is simple about this pathway, but all aspects are fascinating.

### REFERENCES

- Adams, M.D., Ceniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., *et al.* (2000). The genome sequence of *Drosophila melanogaster*. *Science* **28**, 2185–2195.
- Ainscough, M., Bardill, S., Barlow, K., Basham, V., Baynes, C., Beard, L., *et al.* (1998) Genome sequence of the nematode *C. elegans*: platform for investigating biology. *Science* **282**, 2012–2018.
- Aoyama, Y., Funae, Y., Noshiro, M., Horiuchi, T. and Yoshida, Y. (1994) Occurrence of a P450 showing high homology to yeast lanosterol 14-demethylase (P45014DM) in the rat liver. *Biochem. Biophys. Res. Commun.* **201**, 1320–1326.
- Athanikar, J.N. and Osborne, T.F. (1998) Specificity in cholesterol regulation of gene expression by coevolution of sterol regulatory DNA element and its binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4935–4940.
- Bae, S.-H. and Paik, Y.-K. (1997) Cholesterol biosynthesis from lanosterol: development of a novel assay method and characterization of rat liver microsomal lanosterol  $\Delta^{24}$ -reductase. *Biochem. J.* **326**, 609–616.
- Balsten, M. and Byskov, A.G. (1999) Quantitation of meiosis activating sterols in human follicular fluid using HPLC and photodiode array detection. *Biomed. Chromat.* **13**, 382–388.
- Barness, L. (1994) Nutritional requirements of infants and children with respect to cholesterol and related compounds. *Am. J. Med. Genet.* **50**, 353–354.
- Batta, A.K., Salen, G., Tint, G.S. and Shefer, S. (1995) Identification of 19-nor-5,7,9(10)-cholestatrien-3 $\beta$ -ol in patients with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36**, 2413–2418.
- Bellamine, A., Mangla, A.T., Nes, W.D. and Waterman, M.R. (1999) Characterization and catalytic properties of the sterol 14 $\alpha$ -demethylase from *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 8937–8942.
- Brown, M.S. and Goldstein, J.L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331–340.



- Brown, M.S. and Goldstein, J.L. (1998) Sterol regulatory element binding proteins (SREBPs): controllers of lipid synthesis and cellular uptake. *Nutrit. Rev.* **56**, S1-S3.
- Brown, M.S. and Goldstein, J.L. (1999) A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11041–11048.
- Brown, M.S., Ze, J. and Goldstein, J.L. (2000) Regulated Intramembrane proteolysis: a control mechanism conserved from bacteria to human. *Cell* **100**, 391–398.
- Buttke, T. and Folks, T. (1992) Complete replacement of membrane cholesterol with 4', 4'-14-trimethyl sterols in a human T cell line defective in lanosterol demethylation. *J. Biol. Chem.* **267**, 8819–8826.
- Byсков, A.G., Andersen, C.Y., Nordholm, L., Thøgersen, H., Guoliang, X., Wassman, O., Guddal, J.V.A.E. and Roed, T. (1995) Chemical structure of sterols that activate oocyte meiosis. *Nature* **374**, 559–562.
- Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H. and Beachy, P.A. (1996) *Nature* **383**, 407–413.
- Clayton, P., Mills, K., Keeling, J. and FitzPatrick, D. (1996) Desmosterolosis: a new inborn error of cholesterol biosynthesis. *Lancet* **348**, 404.
- Clayton, P.T. (1998) Disorders of cholesterol biosynthesis. *Arch. Dis. Child.* **78**, 185–189.
- Debeljak, N., Horvat, S., Lee, M. and Rozman, D. (2000) Characterization of the mouse lanosterol 14 $\alpha$ -demethylase (Cyp51), a member of the evolutionarily most conserved cytochrome P450 family. *Arch. Biochem. Biophys.* **379**, 37–45.
- Derry, J.M., Gormally, E., Means, G.D., Meindl, A., Kelley, R.I., Boyd, Y. and Herman, G.E. (1999) Mutations in a  $\Delta^8$ - $\Delta^7$ -sterol isomerase in the tattered mouse and X-linked dominant chondrodysplasia punctata. *Nat. Genet.* **22**, 286–290.
- Fitzky, B.U., Witsch-Baumgartner, M., Erdel, M., Lee, J.N., Paik, Y.-K., Glossmann, H., Utermann, G. and Moebius, F. (1998) Mutations in the  $\Delta^7$ -sterol reductase gene in patients with the Smith-Lemli-Opitz syndrome. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8181–8186.
- FitzPatrick, D.R., Keeling, J.W., Evans, M.J., Kan, A.E., Bell, J.E., Porteous, M.E.M., Mills, K., Winter, R.M. and Clayton, P.T. (1998) Clinical phenotype of desmosterolosis. *Am. J. Med. Genet.* **75**, 145–152.
- Fon-Tacer, K., Haugen, T.B., Baltsen, M., Debeljak, N. and Rozman, D. (2002) Tissue-specific transcriptional regulation of the cholesterol biosynthetic pathway leads to accumulation of testis meiosis activation sterol T-MAS. *J. Lipid Res.* **43**, 82–89.
- Gaylor, J.L. and Delwiche, C.V. (1976) Purification of a soluble rat liver protein that stimulates microsomal 4-methyl sterol oxidase activity. *J. Biol. Chem.* **251**, 6638–6645.
- Goldstein, J.L. and Brown, M.S. (1990) Regulation of the mevalonate pathway. *Nature* **343**, 425–430.
- Grinstead, G.F. and Gaylor, J.L. (1982) Total enzymatic synthesis of cholesterol from 4, 4, 14 $\alpha$ -trimethyl-5 $\alpha$ -cholesta-8,24-dien-3 $\beta$ -ol. *J. Biol. Chem.* **257**, 13937–13944.
- Grondahl, C., Lessl, M., Faergem, I., Hegele-Hartung, C., Wasserman, K. and Ottesen, J.L. (2000) Meiosis-activating sterol-mediated resumptions of meiosis in mouse oocytes *in vitro* is influenced by protein synthesis inhibition and cholera toxin. *Biol. Reprod.* **62**, 775–780.
- Grondahl, C., Ottesen, J.L., Lessl, M., Faarup, P., Murray, A., Gronvald, F.C., Hegelehartung, C. and Ahnfeltronne, I. (1998) Meiosis-activating sterol promotes resumptions of meiosis in mouse oocytes cultured *in vitro* in contrast to related oxysterols. *Biol. Reprod.* **58**, 1297–1302.
- Holmer, L., Pezhman, A. and Worman, H.J. (1998) The human lamin B receptor/sterol reductase multigene family. *Genomics* **54**, 469–476.
- Honda, A., Shefer, S., Salen, G., Xu, G., Batta, A.K., Tint, S.G., Honda, M., Chen, C.T. and Holick, M.F. (1996) Regulation of the last two enzymatic reactions in cholesterol biosynthesis in

- rats: effects of BM 15.677, cholesterol, cholic acid, lovastatin and their combinations. *Hepatology* **24**, 437–445.
- Horton, J.D., Shimomura, I., Brown, M.S., Hammer, R.E., Goldstein, J.L. and Shimano, H. (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J. Clin. Invest.* **101**, 2331–2339.
- Hyett, J.A., Clayton, P.T., Moscoso, G. and Nicolaides, K.H. (1995) Increased first trimester bnuchal translucency as a prenatal malformation of Smith-Lemli-Opitz syndrome. *Am. J. Med. Genet.* **58**, 374–376.
- Irons, M., Elias, E.R., Salen, G., Tint, G.S. and Batta, A.K. (1994) Defective cholesterol synthesis in Smith-Lemli-Opitz syndrome. *Lancet* **341**, 1414.
- Jbilo, O., Vidal, H., Paul, R., De Nys, N., Bensaid, M., Silve, S., Carazon, P., Davi, D., Galiegue, S., Bourrie, B., Guillemont, J.-C., Ferrara, P., Loison, G., Maffrand, J.-P., Le Fur, G. and Casellas, P. (1997) Purification and characterization of the human SR 31747A-binding protein. *J. Biol. Chem.* **272**, 27107–27115.
- Kawata, S., Trzaskos, J.M. and Gaylor, J.L. (1985) Microsomal enzymes of cholesterol biosynthesis from lanosterol. *J. Biol. Chem.* **260**, 6609–6617.
- Kelley, R.I., Roessler, E., Hennekam, R.C.M., Feldman, L., Kosaki, K., Jones, M.C., Palumbos, J.C. and Muenke, M. (1996) Holoprocencephaly in RHS/Smith-Lemli-Opitz syndrome: does abnormal cholesterol metabolism affect the function of Sonic hedgehog? *Am. J. Med. Genet.* **66**, 478–484.
- Kim, C.-K., Jeon, K.-L., Lim, D.-M., Johng, T.-N., Trzaskos, J.M., Gaylor, J.L. and Paik, Y.-K. (1995) Cholesterol biosynthesis from lanosterol: regulation and purification of rat hepatic sterol 14-reductase. *Biochim. Biophys. Acta* **1259**, 39–48.
- Laden, B.P., Tang, Y., and Porter, T.D. (2000) Cloning, heterologous expression and enzymological characterization of human squalene monooxygenase. *Arch. Biochem. Biophys.* **374**, 381–388.
- Lamb, D.C., Kelly, D.E., Manning, N.J. and Kelly, S.L. (1998) A sterol biosynthetic pathway in *Mycobacterium*. *FEBS Lett.* **437**, 142–144.
- Laoué, L., Dehart, D.B., Hinsdale, M.E., Maeda, N., Tint, S.G. and Sulik, K.K. (1997) Limb, genital, CNS and facial malformations result from gene/environment-induced cholesterol deficiency: further evidence for a link to sonic hedgehog. *Am. J. Med. Genet.* **73**, 24–31.
- Lecain, E., Chenivresse, X., Spagnoli, R. and Pompon, D. (1996) Cloning by metabolic interference in yeast and enzymatic characterization of *Arabidopsis thaliana* sterol  $\Delta^7$ -reductase. *J. Biol. Chem.* **271**, 10866–10873.
- Liangtao, L. and Kaplan, J. (1996) Characterization of yeast methyl sterol oxidase (*ERG25*) and identification of a human homologue. *J. Biol. Chem.* **271**, 16927–16933.
- Liu, X.Y., Dangel, A.W., Kelley, R.I., Zhao, W., Denny, P., Botcherby, M., Cattanach, B., Peters, J., Hunsicker, P.R., Mallon, A.M., Strivens, M.A., Bate, R., Millen, W., Brown, S.D. and Herman, G.E. (1999) The gene mutated in bare patches and striated mice encodes a novel 3 $\beta$ -hydroxysteroid dehydrogenase. *Nat. Genet.* **22**, 182–187.
- Majdič, G., Parvinen, M., Bellamine, A., Harwood, Jr. H.J., Ku, W.W., Waterman, M.R. and Rozman, D. (2000) Lanosterol 14 $\alpha$ -demethylase (CYP51), NADPH-cytochrome P450 reductase and squalene synthase in spermatogenesis: Late spermatids of the rat express proteins needed to synthesize follicular fluid meiosis activating sterol. *J. Endocrinol.* **166**, 463–474.
- Matsushima, M., Inazawa, J., Takahashi, E., Suzumori, K. and Nakamura, Y. (1996) Molecular cloning and mapping of a human cDNA (SC5DL) encoding a protein homologous to fungal sterol-C5-desaturase. *Cytogenet. Cell. Genet.* **74**, 252–254.

- Mills, K., Mandel, H., Montemagno, R., Soothill, P., Gershoni-Baruch, R. and Clayton, P. (1996) First trimester prenatal diagnosis of Smith-Lemli-Opitz syndrome (7-dehydrocholesterol reductase deficiency). *Pediat. Res.* **39**, 816–819.
- Moebius, F.F., Fitzky, B.U., Lee, J.N., Paik, Y.-K. and Glossmann, H. (1998a) Molecular cloning and expression of the human delta7-sterol reductase. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1899–1902.
- Moebius, F.F., Reiter, R.J., Manner, M. and Glossmann, H. (1997) High affinity of sigma 1-binding sites for sterol isomerization inhibitors: evidence for a pharmacological relationship with the yeast sterol C8-C7 isomerase. *Brit. J. Pharmacol.* **121**, 1–6.
- Moebius, F.F., Striessing, J. and Glossmann, H. (1998b) The mysteries of sigma receptors: new family members reveal a role in cholesterol synthesis. *TIPS* **18**, 67–70.
- Noshiro, M., Aoyama, Y., Kawamoto, T., Gotoh, O., Horiuchi, T. and Yoshida, Y. (1997) Structural and evolutionary studies on sterol 14 $\alpha$ -demethylase P450 (CYP51), the most conserved monooxygenase: I. structural analyses of the gene and multiple sizes of mRNA. *J. Biochem.* **122**, 1114–1121.
- Opitz, J.M. (1994) RSH/SLO (“Smith-Lemli-Opitz”) syndrome: historical, genetic and developmental considerations. *Am. J. Med. Genet.* **50**, 344–346.
- Pahl, H.L. (1999) Signal transduction from endoplasmic reticulum to the cell nucleus. *Physiol. Rev.* **79**, 683–701.
- Porter, J.A., Zoung, K.E. and Beachy, P.A. (1996) Cholesterol modification of hedgehog signalling proteins in animal development. *Science* **274**, 255–259.
- Rozman, D. (2000) Lanosterol 14 $\alpha$ -demethylase (CYP51): a cholesterol biosynthetic enzyme involved in production of meiosis activating sterols in oocytes and testis—a minireview. *Pflügers Arch.—Eur. J. Physiol.* **439** (Suppl.), R56–R57.
- Rozman, D., Fink, M., G.-M., F., Sassone-Corsi, P. and Waterman, M.R. (1999) Cyclic adenosine 3', 5'-monophosphate (cAMP)/cAMP-responsive element modulator (CREM)-dependent regulation of cholesterologenic lanosterol 14 $\alpha$ -demethylase in spermatids. *Mol. Endocrinol.* **13**, 1951–1962.
- Rozman, D., Strömstedt, M., Tsui, L.-C., Scherer, S.W. and Waterman, M.R. (1996a) Structure and mapping of the human lanosterol 14 $\alpha$ -demethylase gene (CYP51) encoding the cytochrome P450 involved in cholesterol biosynthesis: comparison of exon/intron organization with other mammalian and fungal CYP genes. *Genomics* **38**, 371–381.
- Rozman, D., Strömstedt, M. and Waterman, M.R. (1996b) The three human cytochrome P450 lanosterol 14 $\alpha$ -demethylase (CYP51) genes reside on chromosomes 3, 7 and 13: structure of the two retrotransposed pseudogenes, association with a LINE-1 element and evolution of the human CYP51 family. *Arch. Biochem. Biophys.* **333**, 466–474.
- Rozman, D. and Waterman, M.R. (1998) Lanosterol 14 $\alpha$ -demethylase (CYP51) and spermatogenesis. *DrugMetab. Disp.* **26**, 1199–1201.
- Sakai, J., Dunca, E.A., Rawson, R.B., Hua, X., Brown, M.S. and Goldstein, J.L. (1996) Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. *Cell* **85**, 1037–1046.
- Sakakibara, J., Watnabe, R., Yohinori, K. and Ono, T. (1995) Molecular cloning and expression of rat squalene epoxidase. *J. Biol. Chem.* **270**, 17–20.
- Salen, G., Shefer, S., Batta, A.K., Tint, G.S., Xu, G. and Honda, A. (1996a) Abnormal cholesterol biosynthesis in sitosterolemia and the Smith-Lemli-Opitz syndrome. *J. Inherit. Metab. Dis.* **19**, 391–400.
- Salen, G., Shefer, S., Batta, A.K., Tint, G.S., Xu, G., Honda, A., Irons, M. and Elias, E.R. (1996b) Abnormal cholesterol biosynthesis in the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **37**, 1169–1180.

- Shimano, H., Horton, J., Hammer, R.E., Shimomura, I., Brown, M.S. and Goldstein, J.L. (1996) Over production of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J. Clin. Invest.* **98**, 1575–1584.
- Shimano, H., Horton, J.D., Shimomura, I., Hammer, R.E., Brown, M.S. and Goldstein, J.L. (1997a) Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice and culture cells. *J. Clin. Invest.* **99**, 846–854.
- Shimano, H., Shimomura, I., Hammer, R.E., Herz, J., Goldstein, J.L. and Brown, M.S. (1997b) Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for the targeted disruption of the SREBP-1 gene. *J. Clin. Invest.* **100**, 2115–2124.
- Shimomura, I., Shimano, H., Horton, J.D., Goldstein, J.L. and Brown, M.S. (1997) Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *J. Clin. Invest.* **99**, 838–845.
- Silve, S., Dupuy, P.H., Ferrara, P. and Loison, G. (1998) Human lamin B receptor exhibits sterol C14-reductase activity in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1392**, 233–244.
- Silve, S., Dupuy, P.H., Labit-Leboutellier, C., Kaghad, M., Chalon, P., Raiher, A., Taton, M., Lupker, J., Shire, D. and Loison, G. (1996) Emopamil-binding protein, a mammalian protein that binds a series of structurally diverse neuroprotective agents, exhibits  $\Delta^8$ - $\Delta^7$  sterol isomerase activity. *J. Biol. Chem.* **37**, 22434–22440.
- Sloane, D.L., So, O.-Y., Leung, R., Scarafia, L.E., Saldou, N., Jarnagin, K. and Swinney, D.C. (1995) Cloning and functional expression of the cDNA encoding rat lanosterol 14 demethylase. *Gene* **161**, 243–248.
- Smith, D.W., Lemli, L. and Opitz, J.M. (1964) A newly organized syndrome of multiple congenital anomalies. *J. Pediatr.* **64**, 210–217.
- Strömstedt, M., Rozman, D. and Waterman, M.R. (1996) The ubiquitously expressed human CYP51 encodes lanosterol 14 $\alpha$ -demethylase, a cytochrome P450 whose expression is regulated by oxysterols. *Arch. Biochem. Biophys.* **329**, 73–81.
- Strömstedt, M., Waterman, M.R., Haugen, T.B., Tasken, K., Parvinen, M. and Rozman, D. (1998) Elevated expression of lanosterol 14 $\alpha$ -demethylase (CYP51) and the synthesis of oocyte meiosis-activating sterols in postmeiotic germ cells of male rats. *Endocrinology* **139**, 2314–2321.
- Taton, M., Salmon, F., Pascal, S. and Rahier, A. (1994) Plant sterol biosynthesis: recent advances in the understanding of oxidative demethylations at C4 and C14. *Plant Physiol. Biochem.* **32**, 751–760.
- Tint, G.S., Abuelo, D., Till, M., Cordier, M.P., Batta, A.K., Shefer, S., Honda, A., Honda, M., Xu, G.R., Irons, M., Elias, E.R. and Salen, G. (1998) Fetal Smith-Lemli-Opitz syndrome can be detected accurately and reliably by measuring amniotic fluid dehydrocholesterols. *Prenat. Diagn.* **18**, 651–658.
- Tint, S.G., Irons, M., Elias, M.R., Batta, A.K., Frieden, R., Chen, T.S. and Salen, G. (1994) Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330**, 107–113.
- Wassif, C.A., Maslen, C., Kachilelelinjewile, S., Lin, D., Linck, L.M., Connor, W.E., Steiner, R.D. and Porter, F.D. (1998) Mutations in the human sterol  $\Delta^7$ -reductase gene at 11q12–13 cause Smith-Lemli-Opitz syndrome. *Am. J. Hum. Genet.* **63**, 55–62.
- Waterham, H.R., Koster, J., Romeijn, G.J., Hennekam, R.C., Andersson, H.C., Fitzpatrick, D.R., Kelley, R.I. and Wanders, R.J. (2001) Mutations in the  $3\beta$ -hydroxy sterol  $\Delta^{2+}$ -reductase gene causes desmosterolosis, an autosomal recessive disorder of cholesterol triosynthesis. *Am. J. Hum. Genet.* **69**, 685–694.
- Witsch-Baumgartner, M., Fitzky, U., Ogorelkova, M., Kraft, H.G., Moebius, F.F., Glossmann, H., Seedorf, U., Gillesse-Kaesbach, G., Hoffmann, G.F., Clayton, P., Kelley, R.I., and

- Utermann, G. (2000) Mutational spectrum in the  $\Delta^7$ -sterol reductase gene and genotype-phenotype correlation in 84 patients with Smith-Lemli-Opitz Syndrome. *Am. J. Hum. Genet.* **66**, 402–412.
- Wolf, C., Chevy, F., Pham, J., Kolf-Claw, M., Citadelle, D., Mullinez, N. and Roux, C. (1996) Changes in serum sterols of rats treated with 7-dehydrocholesterol- $\Delta^7$ -reductase inhibitors: comparison to levels in humans with Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **37**, 1325–1333.
- Yokoyama, C., Wang, X., Briggs, M.R., Admon, A., Wu, J., Hua, X., Goldstein, J.L. and Brown, M.S. (1993) SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell* **75**, 187–197.

### 3.

## VITAMIN D BIOSYNTHESIS AND ITS DISORDERS

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Vitamin D is a secosteroid derived from cholesterol by opening of the B ring. A series of mitochondrial P450 enzymes bio-activate vitamin D to 1,25(OH)<sub>2</sub>D, which then functions as a classical steroid hormone by binding to a nuclear, zinc-finger receptor. The 25-hydroxylation of vitamin D to 25-hydroxylation vitamin D (25OHD) in the liver is rapid and not regulated, so that 25OHD is the most abundant metabolite in blood, at 10<sup>7</sup> M. Renal 1 $\alpha$ -hydroxylation of 25OHD to 1,25 dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) is rate-limiting and highly regulated, so that most physiologic and genetic attention has been directed to the 1 $\alpha$ -hydroxylase. After decades of effort, four groups succeeded in cloning this enzyme, P450c1 $\alpha$  (also known as CYP27B), in late 1997. Mutations in P450c1 $\alpha$  cause vitamin D-dependent rickets type I, a rare and severe disorder of calcium metabolism which can be treated successfully with oral replacement of 1 $\alpha$ -hydroxylated metabolites of vitamin D. The cloning of the gene for P450c1 $\alpha$  should rapidly advance knowledge of the physiologic regulation of its transcription.

KEY WORDS: vitamin D, P450c1 $\alpha$ , CYP27A1, CYP27B1, 1 $\alpha$ -hydroxylase, rickets, kidney.

### THE VITAMIN D BIOSYNTHETIC PATHWAY

Vitamin D is one of the principal hormonal regulators of calcium and phosphorus metabolism and is critically important for normal growth and mineralization of bone. Vitamin D is frequently overlooked by the steroid community because it is a secosteroid, having an opened B ring, and probably also because it is not produced by one of the "traditional" steroidogenic tissues. Nevertheless it shares all essential characteristics with the more conventional steroids produced in the adrenals, gonads and elsewhere: (1) it is produced from cholesterol by a multistep biosynthetic pathway; (2) the pathway consists of at least three cytochrome P450 enzymes that are genetically related to those found in the adrenal and gonad; and (3) the final active hormone product, 1,25(OH)<sub>2</sub>D, binds to a typical zinc-finger nuclear receptor to elicit its principal activities by activating the transcription of specific genes. 1,25(OH)<sub>2</sub>D stimulates calcium and phosphorus absorption from the intestine, thus maintaining their circulating concentrations at appropriate levels for normal

growth and mineralization of bone. Vitamin D also acts on bone, kidney, parathyroid gland and on other tissues unrelated to bone and mineral metabolism. Thus the genetics of vitamin D biosynthesis and bio-activation are of central interest to studies of steroidogenesis, growth, mineral metabolism and tissue differentiation.

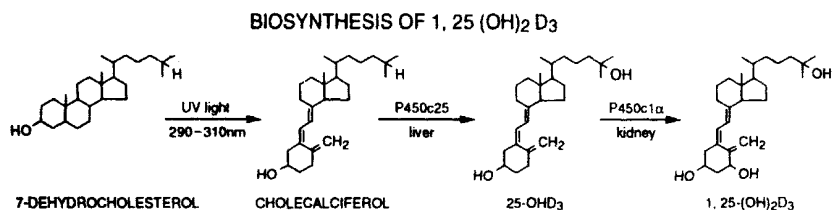
Vitamin D exists in two forms: ergocalciferol (vitamin D<sub>2</sub>) produced by plants, and cholecalciferol (vitamin D<sub>3</sub>) produced by animal tissues and by the action of near-ultraviolet radiation (290–320 nm) on 7-dehydrocholesterol (provitamin D<sub>3</sub>) in human skin (Strewler and Rosenblatt, 1995; Feldman *et al.*, 1996). The ultra-violet radiation cleaves the bond between carbons 9 and 10 of the B ring of 7-dehydrocholesterol to form the secosteroid, previtamin D<sub>3</sub>, which then isomerizes over a period of hours to vitamin D<sub>3</sub>. Prolonged excessive exposure to sunlight forms the biologically inactive photoisomers lumisterol and tachysterol, which may protect against the development of vitamin D intoxication (Holick *et al.*, 1981). Vitamin D<sub>3</sub> binds to the vitamin D binding protein, a58kDa plasma  $\alpha$ -globulin, synthesized primarily in the liver, and leaves the skin to enter the circulation. Vitamin D<sub>2</sub> differs from D<sub>3</sub> only in having a double bond between carbons 22 and 23 on the side chain, and having an additional methyl group attached to carbon 24 (Figure 3.1). For convenience, we shall generally dispense with the distinction between D<sub>2</sub> and D<sub>3</sub>.

Vitamin D itself is a biologically inactive pro-hormone. In order to acquire the ability to bind to and activate the vitamin D receptor, and thus exhibit biological activity, vitamin D must first undergo two successive hydroxylations at carbons #25 and #1 (Figure 3.1). The 1,25-dihydroxy metabolites of vitamins D<sub>2</sub> and D<sub>3</sub> have equivalent capacity to bind to the vitamin D receptor and have equal biological potency. The 25-hydroxylation of vitamin D occurs primarily in the liver. There appear to be active but distinct 25-hydroxylases in both the endoplasmic reticulum and the mitochondria, although the mitochondrial enzyme (P450c25, also known as CYP27A1) appears to be the physiologically more important. This mitochondrial cytochrome P450 enzyme apparently also hydroxylates carbons 26 and 27 of cholesterol to initiate bile acid synthesis in the liver (see below). The 25-hydroxylase activity in the liver is so abundant that about 50% of circulating vitamin D is converted to 25-hydroxyvitamin D (25OHD) on a single pass through the liver (Gascon-Barrae and Gamache, 1991). The activity of this enzyme is not under tight physiologic regulation, and the circulating concentration of 25OHD, about  $10^{-7}$  M, is determined primarily by dietary intake of vitamin D and amount of sunlight exposure. Although 25OHD is the most abundant form of vitamin D in the blood, like vitamin D itself, it has minimal capacity to bind to the vitamin D receptor and elicit a biologic response.

The active form of vitamin D, 1,25 dihydroxyvitamin D [1,25 (OH)<sub>2</sub>D], is formed by the 1 $\alpha$ -hydroxylation of 25OHD. The 1 $\alpha$ -hydroxylase enzyme has long been thought to be confined to the proximal convoluted tubules of the kidney, and circulating concentrations of 1,25(OH)<sub>2</sub>D primarily reflect its renal synthesis; however, 1 $\alpha$ -hydroxylase activity also is found in keratinocytes, macrophages and osteoblasts. Renal 1 $\alpha$ -hydroxylase activity is tightly regulated by parathyroid hormone (PTH), calcium, phosphorus and 1,25(OH)<sub>2</sub>D itself (Kumar, 1984; Breslau, 1988). Because this is the hormonally regulated, rate-limiting, bio-

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**Figure 3.1 Biosynthesis of vitamin D<sub>3</sub>.** Ultraviolet light (290 to 310nm) cleaves the B ring of 7-dehydrocholesterol in the skin to yield cholecalciferol (vitamin D<sub>3</sub>). Vitamin D, which circulates in blood bound to 56 kDa vitamin D-binding protein, is taken up by the liver, where it is 25-hydroxylated. The resulting 25-OHD, which is the most abundant form of vitamin D in the human circulation, may undergo 1 $\alpha$ -hydroxylation by P450c1 $\alpha$  to yield the active hormonal compound 1, 25 (OH)<sub>2</sub>D.

activating step, and because this enzyme is commonly disrupted in acquired renal disease as well as in a rare genetic disease, it has been the subject of intense study for nearly 30 years. Despite intensive efforts, the 1 $\alpha$ -hydroxylase enzyme has not been isolated by conventional protein biochemistry. However, the second half of 1997 witnessed the cloning of the cDNA and gene for this enzyme from rodents and human beings, leading to an understanding of its genetics, enzymology and transcriptional regulation.

Another important vitamin D-metabolizing enzyme, the 25-hydroxyvitamin D-24-hydroxylase (P450c24, also known as CYP24), is found in kidney, intestine, lymphocytes, fibroblasts, bone, skin, macrophages and possibly other tissues (Armbrrecht *et al.*, 1992). The enzyme can convert 25OHD to 24,25(OH)<sub>2</sub>D and 1,25(OH)<sub>2</sub>D to 1,24,25(OH)<sub>3</sub>D; these reactions appear to be the first steps in the metabolic inactivation and excretion of either 25OHD or 1,25(OH)<sub>2</sub>D. The kidney and intestine are major sites of hormonal inactivation of vitamin D by virtue of their abundant 24-hydroxylase activity.

#### THE VITAMIN D BIOSYNTHETIC ENZYMES ARE MITOCHONDRIAL FORMS OF CYTOCHROME P450

The vitamin D 25-hydroxylase, the 1 $\alpha$ -hydroxylase and the bio-inactivating 24-hydroxylase are cytochrome P450 enzymes found in mitochondria. All cytochrome P450 enzymes function as oxidases using electrons from NADPH and molecular oxygen (Nebert and Gonzalez, 1987; Black and Coon, 1987). There are two forms of cytochrome P450 enzymes. Type I P450s are found in mitochondria and in bacteria. Eukaryotic type I enzymes include the cholesterol side-chain cleavage enzyme, P450<sub>scc</sub> (CYP11A) (Chung *et al.*, 1986; Morohashi *et al.*, 1987), and the two isozymes of CYP11B, CYP11B1 (11 $\beta$ -hydroxylase) and CYP11B2 (aldosterone synthase) (Mornet *et al.*, 1989; see also [Chapter 6](#)). Type I enzymes are also found in many prokaryotes, including three soluble forms of P450 whose structures have been solved by x-ray crystallography: P450<sub>cam</sub> (CYP101) from *Pseudomonas putida* (Poulos *et al.*, 1986, 1987), P450<sub>terp</sub> (CYP108) from *Pseudomonas* species (Hasemann *et al.*, 1994) and P450<sub>eryF</sub> (CYP107A1) from the activo-mycete *Saccharopolyspora erythraea* (Cupp-Vickery and Poulos, 1995). The type I P450 enzymes



receive electrons from NADPH via the intermediacy of an electron-transfer chain consisting of a flavoprotein and an iron-sulfur protein. The flavoprotein, termed ferredoxin reductase (Solish *et al.*, 1988; Lin *et al.*, 1990), is bound to the inner mitochondrial membrane, where it receives electrons from NADPH and then transfers them to an iron-sulfur protein termed ferredoxin (Chang *et al.*, 1988; Picado-Leonard *et al.*, 1988), which may be loosely associated with the inner membrane or may be in solution in the mitochondrial matrix (Privalle *et al.*, 1983; Hanukoglu *et al.*, 1990). Ferredoxin then interacts with one of a variety of different P450 moieties, which bind the substrate, receive electrons and molecular oxygen, catalyze the reaction through the intermediacy of a heme group and release the product. The tissue distribution of the expression of the P450 moieties is highly specific, but human ferredoxin (Picado-Leonard *et al.*, 1988) and ferredoxin reductase (Brentano *et al.*, 1992) expressed in all tissues, albeit in highly varying quantities. Type II cytochrome P450 enzymes, such as the hepatic enzymes involved in drug metabolism or the steroidogenic enzymes P450c17, P450arom and P450c21 (a.k.a. CYP17, CYP19 and CYP21) are found in the endoplasmic reticulum, where they receive electrons from a flavoprotein termed NADPH-P450 oxidoreductase; in some cases, electron transfer from P450 oxidoreductase is allosterically facilitated by cytochrome  $b_5$  (Auchus *et al.*, 1998; Geller *et al.*, 1999). P450 oxidoreductase is quite different from ferredoxin reductase, and functions without the need of an iron/sulfur intermediate such as ferredoxin. Thus mitochondrial (Type I) and microsomal (Type II) P450 enzymes are substantially different genetically and enzymologically.

#### VITAMIN D 25-HYDROXYLASE

Initial work indicated that the vitamin D 25-hydroxylase was a microsomal enzyme (Bhattacharyya and Deluca, 1974), and subsequently work indicated it was mitochondrial (Bjorkhem and Holmberg, 1978). The subcellular location of each of the vitamin D-metabolizing enzymes remained somewhat controversial until each was cloned, and this controversy remains with 25-hydroxylase. The first vitamin D-metabolizing enzyme to be cloned was a 25-hydroxylase, but this work originated with studies of bile acid 26-hydroxylation. Wikvall identified a mitochondrial P450 isolated from rabbit liver that had 26-hydroxylase activity, termed P450c26 (Wikvall, 1984). Subsequent cloning of the cDNA and its expression in COS-1 cells confirmed the activity of this enzyme (Andersson *et al.*, 1989). Three groups independently used similar techniques to purify a mitochondrial P450 from rat liver that had vitamin D 25-hydroxylase activity, termed P450c25 (Dahlback and Wikvall, 1988; Masumoto *et al.*, 1988; Andersson *et al.*, 1989; Shaiq and Avadhani, 1989). Monoclonal antibodies directed against the 26-hydroxylase did not inhibit 25-hydroxylation of vitamin D, suggesting that these two activities were catalyzed by distinct enzymes (Dahlback and Wikvall, 1988). However, two groups working independently used polyclonal antisera to the vitamin D 25-hydroxylase to screen bacteriophage-based expression libraries of rat liver cDNA, yielding a rat P450c25 cDNA that was 73% identical to rabbit liver P450c26 (Usui *et al.*, 1990; Su *et al.*, 1990). When expressed in transfected cells, this single P450 could catalyze both vitamin D 25-hydroxylation and cholesterol 26-hydroxylation. The corresponding human cDNA was cloned in 1991 (Cali and Russell,

1991). As it can hydroxylate both carbons 26 and 27 of cholesterol, the gene for this enzyme is officially termed CYP27A1, but the enzyme is commonly referred to as P450c25, to connote its hydroxylation of carbon 25.

Little is known about the regulation of 25-hydroxylase by vitamin D. Administration of 1, 25(OH)<sub>2</sub>D<sub>3</sub> to healthy human subjects prevented the usual 2.5-fold increase in serum concentration of 25OHD induced by oral vitamin D, suggesting that hepatic 25-hydroxylation of vitamin D was impaired by exogenously administered 1,25(OH)<sub>2</sub>D (Bell *et al.*, 1984). However, the effect of 1,25(OH)<sub>2</sub>D to reduce serum 25OHD in the rat was due to an increase in the metabolic clearance rate of 25OHD, with no change in its production rate (Halloran *et al.*, 1986; Halloran and Castro, 1989).

Deficiency of cholesterol 27-hydroxylase activity causes cerebrotendinous xanthomatosis (Cali and Russell, 1991; Leitersdorf *et al.*, 1993), and numerous mutations in the CYP27A gene encoding P450c27 have been found in this disease (Garuti *et al.*, 1996). However, it has not been clear whether these patients have a disorder in vitamin D biosynthesis, although they may have osteoporosis and an increased incidence of bone fractures (Berlinger *et al.*, 1992). Furthermore, a patient has been described with apparent vitamin D 25-hydroxylase deficiency who had no evidence of cerebrotendinous xanthomatosis (Casella *et al.*, 1994) and we have found no mutations in this patient's CYP27A gene (C.J.Lin and W.L.Miller, unpublished). Finally, a microsomal P450 that catalyzes 25-hydroxylation of vitamin D<sub>3</sub>, but not vitamin D<sub>2</sub>, has recently been cloned from pig liver (Postlind *et al.*, 1997). This enzyme appears to be the pig homologue of human P4502B6, with which it shares 76% amino acid sequence identity. However, human P4502B6 is not expressed in 5–10% of normal people, and hence cannot be an indispensable enzyme in vitamin D biosynthesis. Thus, while it remains quite possible that the principal enzyme catalyzing cholesterol 26- and 27-hydroxylation and the principal enzyme catalyzing vitamin D 25-hydroxylation are one and the same, it is also possible that there may be multiple 25-hydroxylation enzymes with partially overlapping activities. Therefore, much remains to be determined about the genetics and enzymology of vitamin D 25-hydroxylation. We suggest that there is more than one hepatic enzyme that can catalyze vitamin D 25-hydroxylation, so that the lack of any single one of these will not cause a clinical syndrome of profound 25-hydroxylase deficiency, accounting for the apparent absence of this hypothetical disease.

#### VITAMIN D 24-HYDROXYLASE

The second vitamin D metabolizing enzyme to be cloned was the 24-hydroxylase. This was also cloned by purifying the enzyme from renal mitochondria, raising a polyclonal antiserum, and screening a rat kidney cDNA expression library (Ohyama *et al.*, 1991). This was followed by the cloning of the corresponding rat gene (Ohyama *et al.*, 1993) and by the human cDNA (Chen *et al.*, 1993) and gene (Chen and DeLuca, 1995). Studies with the purified rat renal enzyme (Ohyama *et al.*, 1991) and with cells expressing the transfected human cDNA (Chen *et al.*, 1993) showed that this P450c24 could catalyze the 24-hydroxylation of both 25OHD and 1,25 (OH)<sub>2</sub>D. The cloning of the rat CYP24 gene encoding P450c24, which spans ~15 kb and contains 12 exons, also showed that this gene was a close evolutionary relative of the genes for mitochondrial P450scc (CYP11A) and the two isozymes of CYP11B,

CYP11B1 and CYP11B2, (Ohyama *et al.*, 1991) (for a review of the CYP11A and CYP11B genes, see Fardella and Miller, 1996; White *et al.*, 1994, also Chapters 5 and 7). This provided strong evidence that all of the mitochondrial P450s were closely related, which was then confirmed with the cloning of the  $1\alpha$ -hydroxylase.

In the kidney, PTH decreases (Armbricht *et al.*, 1982) and  $1,25(\text{OH})_2\text{D}_3$  increases 24-hydroxylase activity *in vivo* and in various preparations of renal tissue studied *in vitro* (Armbricht *et al.*, 1992). The suppressive effect of PTH on 24-hydroxylase activity is thought to be mediated via cAMP, and the stimulatory effect of  $1,25(\text{OH})_2\text{D}$  on 24-hydroxylase activity requires synthesis of new protein. The 5' flanking region of the CYP24 gene appears to contain a vitamin D-responsive element (VDRE) and two putative cAMP response elements (Ohyama *et al.*, 1993; Zierold *et al.*, 1994). PTH and  $1,25(\text{OH})_2\text{D}$  regulate P450c24 mRNA abundance (Shinki *et al.*, 1992; Akeno *et al.*, 1997; Armbricht and Boltz, 1991; Chen *et al.*, 1993) and the up-regulation by  $1,25(\text{OH})_2\text{D}$  is due to an increase in gene transcription (Roy *et al.*, 1997).

### VITAMIN D $1\alpha$ -HYDROXYLASE

Despite the cloning of the 25- and 24-hydroxylases in 1990 and 1991, the  $1\alpha$ -hydroxylase, which is the most important enzyme biologically because it is rate-limiting and hormonally regulated, resisted all efforts at protein purification and cloning until quite recently. Then, in the second half of 1997, four independent groups using different approaches reported the cloning of the mouse, rat, and human vitamin D  $1\alpha$ -hydroxylase, P450c1 $\alpha$ . All of the initial reports were submitted for publication within a 3-month period, and hence, all should be regarded as simultaneous achievements (Fu *et al.*, 1997a, b; Monkawa *et al.*, 1997; Shinki *et al.*, 1997a; St-Arnaud *et al.*, 1997; Takayama *et al.*, 1997). Several reasons underlay the difficulty in cloning P450c1 $\alpha$ , the most important one being that there is very little P450c1 $\alpha$  protein in the kidney. Physiologic induction of enzyme activity, and hence presumably of increased amounts of protein was attempted by rearing rats on a D-deficient, low-calcium, low-phosphorus diet in the absence of UV light. However, although this stimulated  $1\alpha$ -hydroxylase activity considerably, efforts to purify the  $1\alpha$ -hydroxylase protein, as had been done previously for the 24- and 25-hydroxylases, were consistently unsuccessful. Thus, the direct protein-based immunologic approaches used to clone P450c24 and P450c25 failed; instead, all four groups succeeded by employing genetic approaches.

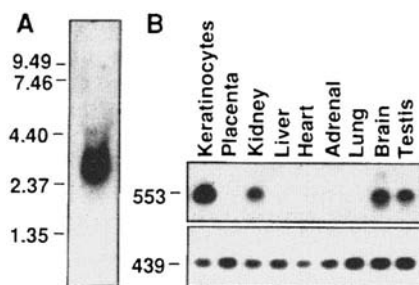
Two groups (Shinki *et al.*, 1997a; St-Arnaud *et al.*, 1997) prepared renal cDNA from rats raised on low calcium, low phosphorus, vitamin D-depleted diets which should stimulate renal  $1\alpha$ -hydroxylase activity while suppressing 24-hydroxylase activity. Glorieux's laboratory made a cDNA library and screened it with a fragment of P450c24 cDNA corresponding to the generally well-conserved heme-binding site, yielding a putative P450c1 $\alpha$  clone whose identity as a  $1\alpha$ -hydroxylase was confirmed by the activity of the expressed protein (St-Arnaud *et al.*, 1997). Suda's laboratory prepared degenerate-sequence oligonucleotides based on the conserved ferredoxin-binding sites and heme-binding sites of CYP24 and P450c25 and used these for PCR amplification of the rat renal cDNA. This PCR product was then cloned, yielding partial-length candidate clones for P450c1 $\alpha$  which were

then used to screen a full-length rat renal cDNA library identifying the P450c1 $\alpha$  cDNA (Shinki *et al.*, 1997a).

Kato's laboratory solved the problem of increasing P450c1 $\alpha$  expression by preparing renal cDNA from mice that were homozygous for a vitamin D receptor (VDR) knockout (VDR-/-) (Takayama *et al.*, 1997). In the absence of a functional VDR, these animals cannot respond to 1,25(OH) $_2$ D. Because they fail to respond to 1,25(OH) $_2$ D, these animals behave as though they were profoundly deficient in 1,25(OH) $_2$ D, and hence attempt to compensate by over-producing 1,25(OH) $_2$ D through a 10-fold increase in the synthesis of the rate-limiting enzyme, P450c1 $\alpha$ . Renal cDNA from these animals was cloned into cells that also expressed the ligand-binding domain of VDR connected to a yeast GAL4 DNA-binding domain. These cells were also transfected with vectors for the ferredoxin and ferredoxin reductase needed by CYP27B1 and with a vector with *lac Z* regulated by GAL4, thus providing a vitamin D-based selection system. Thus, when the cells were given 25OHD, those expressing P450c1 $\alpha$  could produce 1,25(OH) $_2$ D, thus activating the VDR/GAL4 fusion, which could be determined colorimetrically by induction of the  $\beta$ -galactosidase activity of *lac Z* (Takayama *et al.*, 1997).

Our laboratory solved the problem of the low renal abundance of P450c1 $\alpha$  by turning to a different tissue system, the cultured human keratinocyte (Fu *et al.*, 1997a). Work in the 1980s had shown that primary cultures of human keratinocytes grown in serum-free medium containing very low concentrations of calcium acquire substantial vitamin D 1 $\alpha$ -hydroxylase activity (Bikle *et al.*, 1986a, b, 1993). We reasoned that these cells would give us a source of RNA enriched for P450c1 $\alpha$  in RNA, and had the advantage of permitting us to work directly with a human system, avoiding the need to clone a rodent cDNA first. After several unsuccessful attempts at expression-based cloning, we used several sets of degenerate-sequence primers based on the conserved sequences of the ferredoxin-binding site and heme-binding site to amplify a 300bp fragment from human keratinocyte cDNA. This cDNA had a unique sequence that resembled that of other mitochondrial P450s. The 300bp cDNA was used to screen a human keratinocyte cDNA library, yielding a 1.9kb partial-length cDNA whose 5' end was obtained by primer extension to yield the full-length 2.4kb cDNA (Fu *et al.*, 1997a). The power of this keratinocyte system is illustrated in Figure 3.2, which shows that P450c1 $\alpha$  mRNA is about 100-fold more abundant in cultured keratinocytes than it is in the kidney.

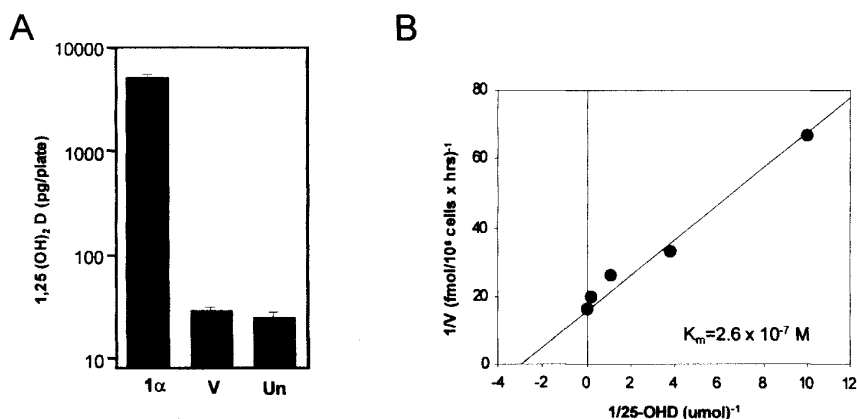
The mouse, rat and human cDNAs prepared by each group were expressed in transfected cell systems, showing that each encoded a protein that could catalyze the conversion of 25OHD to 1,25(OH) $_2$ D, as assayed by high pressure liquid chromatography (HPLC). However, the history of vitamin D chemistry contained many examples of the isolation of sterols that resembled 1,25(OH) $_2$ D chromatographically, but were, in fact, other compounds. Thus an unusually high standard of proof had to be applied to the identification of any putative 1 $\alpha$ -hydroxylase protein or gene and its biosynthesized 1,25(OH) $_2$ D product. Takayama *et al.* (1997) provided biological evidence that the mouse enzyme they had cloned produced 1,25(OH) $_2$ D, because product of their putative P450c1 $\alpha$  clone produced a vitamin D metabolite that biologically transactivated the VDR, both during the cloning process and in promoter/reporter assays. St-Arnaud *et al.* (1997) purified a very small amount (0.4 to 0.8ng) of the putative 1,25(OH) $_2$ D $_3$  produced by their enzyme and showed



**Figure 3.2 Expression of 1 $\alpha$ -hydroxylase mRNA.** (A) Northern blot of 30  $\mu$ g of human keratinocyte total RNA probed with a 340bp *Kpn* I fragment of the cDNA reveals a single band of  $\sim$ 2.5kb. (B) Distribution and abundance of human 1 $\alpha$ -hydroxylase mRNA. cDNA from 1  $\mu$ g of total RNA was PCR-amplified for 30 cycles to yield a 553-bp 1 $\alpha$ -hydroxylase cDNA fragment; a similar procedure was used to amplify a 439bp actin cDNA fragment as control. The PCR products were run on agarose gels, blotted, and probed with P450c1 $\alpha$  (CYP27B1) cDNA (above) or actin cDNA (below). Only 1/25 of the keratinocyte DNA product was loaded, but the band is at least four times more intense than in the kidney sample, indicating at least 100 times greater abundance of P450c1 $\alpha$  mRNA in keratinocyte RNA than in kidney RNA.

by mass spectrometry that it had a very similar mass spectrum to authentic 1,25(OH) $_2$ D $_3$ . In addition, St-Arnaud and colleagues used their rat P450c1 $\alpha$  cDNA to identify a human genomic DNA clone (St-Arnaud *et al.*, 1997). Although this clone was not sequenced, they used fluorescence *in situ* hybridization (FISH) to show that the corresponding human gene lay on chromosome 12q13.1-q13.3, which corresponded to the 12q13-q14 locus they had previously identified for vitamin D-dependent rickets type I (VDDR-I) (also called pseudo vitamin D-deficient rickets, or PDDR), a disease presumed to represent a genetic lesion in renal vitamin D 1 $\alpha$ -hydroxylase activity (Labuda *et al.*, 1990, 1996). This finding provided further indirect evidence that this disease was caused by mutations of P450c1 $\alpha$ , as had long been suspected on clinical and hormonal grounds (Fraser *et al.*, 1973).

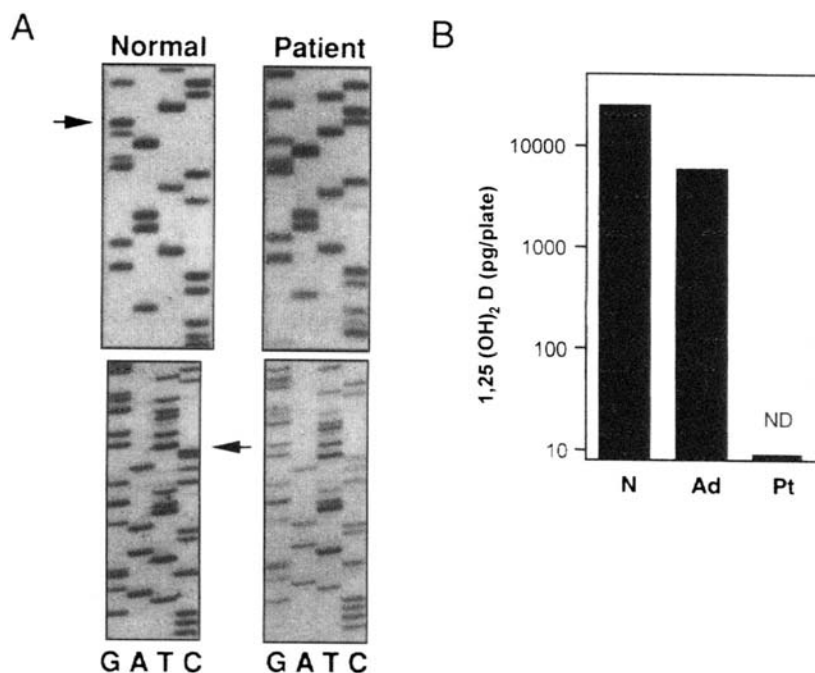
Because we had cloned P450c1 $\alpha$  from keratinocytes instead of kidney, we had to provide compelling evidence that the keratinocyte P450 enzyme we had cloned was identical to the renal 1 $\alpha$ -hydroxylase, rather than being an unrelated keratinocyte-specific P450 that had 1 $\alpha$ -hydroxylase activity. Therefore, we provided four lines of evidence that the keratinocyte and renal enzymes are encoded by the same gene (Fu *et al.*, 1997a). These studies required fairly substantial amounts of 1,25(OH) $_2$ D produced by our cloned, recombinant P450c1 $\alpha$  enzyme; production of large amounts of 1,25(OH) $_2$ D was possible because we expressed our cloned P450c1 $\alpha$  cDNA in mouse Ley dig MA-10 cells. MA-10 cells normally produce large amounts of steroid hormones and hence contain abundant ferredoxin and ferredoxin reductase to support the activity of their endogenous P450 $_{sc}$  (CYP11A). We had previously shown that transfection of cDNAs for mitochondrial CYP11B2 yields 100- to 1000-fold more activity than when CYP11B2 is expressed in COS-1 cells (Fardella *et al.*, 1996). Using this system, we first showed that our keratinocyte clone encoded an enzyme that catalyzed the conversion of 25OHD to 1,25(OH) $_2$ D, as measured both by a radioreceptor assay and by co-migration with authentic 1,25(OH) $_2$ D standards by HPLC



**Figure 3.3 Expression of the keratinocyte P450c1 $\alpha$  cDNA in MA-10 cells.** (A) Activity, expressed as picogram of 1,25(OH)<sub>2</sub>D produced per 10cm plate of cells; the logarithmic y-axis begins at 8pg, the detection limit of the radioreceptor assay. MA-10 cells have a minimal endogenous level of 1 $\alpha$ -hydroxylase activity, shown by the level in untransfected cells (Un) or vector-transfected cells (V) of ~25pg/plate; by contrast, cells expressing the P450c1 $\alpha$  cDNA produced ~5000pg/plate. (B) Lineweaver-Burke plot of 1 $\alpha$ -hydroxylase activity of P450c1 $\alpha$  expressed in MA-10 cells, showing a  $K_m$  of  $\sim 2.6 \times 10^{-7} \text{ M}$ .

using two different solvent systems. Our putative P450c1 $\alpha$  had a  $K_m$  for 25OHD of  $2.7 \times 10^{-7} \text{ M}$ , which is similar to the circulating concentrations of the 25OHD substrate (Figure 3.3). The increased 1 $\alpha$ -hydroxylase activity we achieved with MA-10 cells permitted us to prepare biochemically useful amounts (230ng) of the 1,25(OH)<sub>2</sub>D product and to prove its complete chemical identity by gas chromatography/mass spectrometry of the trimethylsialated derivative; this provided definitive chemical proof that our enzyme produced authentic 1,25(OH)<sub>2</sub>D, and not some other related metabolite of vitamin D. Third, we used reverse transcription/polymerase chain reaction (RT-PCR) to show that the same sequences we had cloned from keratinocytes were expressed in the human kidney, as well as in other tissues. Finally, we provided direct genetic proof of the identity of our P450c1 $\alpha$  by showing that the gene encoding the keratinocyte P450c1 $\alpha$  was the same gene that encoded the renal enzyme. This was done by studying a patient with VDDR-I, which constitutes the human P450c1 $\alpha$  gene knockout of nature. We obtained keratinocytes from a patient with VDDR-I and showed that these keratinocytes lacked 1 $\alpha$ -hydroxylase activity. We cloned the P450c1 $\alpha$  cDNA from these cells, and found that the patient was a compound heterozygote for two premature translation termination (non-sense) mutations (Figure 3.4). This provided unambiguous genetic proof that the keratinocyte and renal P450c1 $\alpha$  enzymes are encoded by the same gene, and provided the first definitive proof that VDDR-I is caused by mutations in P450c1 $\alpha$  (Fu *et al.*, 1997a).

Follow-up papers appeared rapidly in late 1997 and early 1998. Suda's laboratory used their rat P450c1 $\alpha$  cDNA to clone a human cDNA and gene (Monkawa *et al.*, 1997). Kato's laboratory cloned the human cDNA and gene, and confirmed that patients with VDDR-I have mutations in P450c1 $\alpha$  (Kitanaka *et al.*, 1998). After cloning of the human cDNA, we



**Figure 3.4 Mutation of P450c1 $\alpha$  causes VDDR-I.** (A) Keratinocytes from a normal person (left) and from a patient with VDDR-I (right) were used to prepare mRNA, which was then reverse-transcribed, and the P450c1 $\alpha$  cDNA was PCR-amplified with specific primers. The upper panel displays normal and patient cDNA sequenced in the region of codon 211, showing that a normal G (arrow) is deleted in the patient; the lower panel displays the region of codon 231, showing a normal C (arrow) is deleted in the patient. The patient was thus a compound heterozygote for two base deletion/frameshift mutations, each of which was confirmed in genomic DNA as each created a novel restriction endonuclease site (*Bsu* 361 and *Tsp* 451 for codons 211 and 231, respectively) (Fu *et al.*, 1997a). (B) 1 $\alpha$ -hydroxylase activity of keratinocytes from human neonatal foreskin (N), adult skin (Ad), and skin from a patient with VDDR-I (Pt). The scale is logarithmic and begins at the level of detection of the assay. No activity was detected in the patient.

reported the cloning and complete sequence of the human gene, its localization to chromosome 12 by somatic cell hybrid analysis, the localization of the transcriptional start site, and a structural analysis of the genes for the four mitochondrial P450s now available (P450c1 $\alpha$ , P450c24, P450sc, P450c11) (Fu *et al.*, 1997a, b) and more recently we examined the genetics of VDDR-I in 17 families (Wang *et al.*, 1998) and we have described the first mutations that retain partial 1 $\alpha$ -hydroxylase activity *in vitro* (Wang *et al.*, 2002).

The cloning of P450c1 $\alpha$  has permitted early insights into its molecular regulation. In the kidney, 1 $\alpha$ -hydroxylase activity is stimulated by PTH, insulin-like growth factor 1 (IGF-1), hypocalcemia, and hypophosphatemia; and activity is suppressed by hypercalcemia, hyperphosphatemia, and 1,25(OH)<sub>2</sub>D3 itself (Feldman *et al.*, 1996; Fraser, 1980; Reichel *et al.*, 1989). The stimulatory effect of PTH on 1 $\alpha$ -hydroxylase activity can be mimicked by

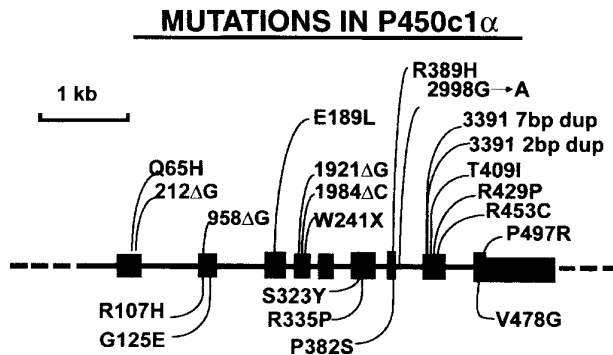
cAMP and forskolin, suggesting that activation of the protein kinase A signaling pathway is involved; such stimulation also requires an intact phospholipase C/protein kinase C signaling pathway (Janulis *et al.*, 1993; Henry, 1986). The abundance of P450c1 $\alpha$  mRNA is regulated by PTH, low calcium diet, and 1,25(OH) $_2$ D in intact animals (St-Arnaud *et al.*, 1997; Shinki *et al.*, 1997b). The mouse CYP27B1 promoter was transcriptionally activated by PTH and forskolin and although 1,25(OH) $_2$ D $_3$  did not induce a decrease in basal promoter activity, it did inhibit by ~40% the increase otherwise induced by PTH, even though these investigators could not identify an apparent VDRE sequence in 1.7kb of 5' flanking DNA (Brenza *et al.*, 1998). Two laboratories also found that transcription of the human CYP27B1 gene is induced by PTH, forskolin and calcitonin (Murayama *et al.*, 1998; Kong *et al.*, 1990). Murayama *et al.* (1998) reported suppression of basal promoter activity by 1,25(OH) $_2$ D, but Kong *et al.* (1990) observed instead that 1,25(OH) $_2$ D $_3$  suppressed PTH-stimulated promoter activity, similar to the findings of Brenza and colleagues (Brenza *et al.*, 1998).

Phosphorus is an important regulator of the renal 1,25(OH) $_2$ D production. In experimental animals (Tanaka and DeLuca, 1973; Hughes *et al.*, 1975; Baxter and DeLuca, 1976; Booth *et al.*, 1985) and healthy human subjects (Gray *et al.*, 1977; Maierhofer *et al.*, 1984; Portzle *et al.*, 1986, 1987, 1989) hypophosphatemia induced by restricting dietary phosphorus increases the production and serum concentration of 1,25(OH) $_2$ D and in 1 $\alpha$ -hydroxylase activity. The changes in serum 1,25(OH) $_2$ D concentrations induced by manipulating dietary phosphorus are due to changes in 1,25(OH) $_2$ D production, as its metabolic clearance does not change (Portale *et al.*, 1986). Hypophosphatemia induced by restricting dietary phosphorus in normal mice induced a 5-fold increase in both renal 1 $\alpha$ -hydroxylase activity and P450c1 $\alpha$  mRNA abundance, while renal 24-hydroxylase mRNA abundance decreased 3-fold (Zhang *et al.*, 2002). Thus phosphorus regulates renal expression of P450c1 $\alpha$  and P450c24 reciprocally. The stimulation of 1,25(OH) $_2$ D production by phosphorus restriction is abolished by hypophysectomy and is restored by administration of growth hormone or IGF-I (Gray, 1981; Gray and Garthwaite, 1985; Halloran and Spencer, 1988), indicating that an intact growth hormone/IGF-I axis is required. Growth hormone and IGF-I enhance renal tubular reabsorption of phosphate; but it is not known how tubular transport of phosphate might be linked to regulation of P450c1 $\alpha$ .

### The molecular genetics of 1 $\alpha$ -hydroxylase deficiency (VDDR-I)

Initial genetic studies of VDDR-I began well before the cloning of P450c1 $\alpha$  from any species. The search for the gene that is mutated in VDDR-I was facilitated by the very high incidence of this disease among relatively inbred French-Canadian populations. Using linkage analysis to RFLP markers, Labuda and colleagues mapped the responsible gene to chromosome 12q14 (Labuda *et al.*, 1990). In a follow-up study, these investigators used microsatellite analysis to show that the French-Canadian VDDR-I population resulted from two independent founder mutations, one in the "Charlevoix" region of Quebec and the other in the Eastern Canadian "Acadian" population. Examination of 11 different microsatellite markers spanning 12 centimorgans demonstrated very tight linkage to





**Figure 3.5** Map of the human gene encoding P450c1 $\alpha$ , as reported by Fu *et al.* (1997b). All mutations causing 1 $\alpha$ -hydroxylase deficiency reported through mid-1999 are shown.

D12S90, and permitted the haplotyping of all patients with only three markers—D12S90, D12S305 and DRS104 (Labuda *et al.*, 1996). Although, this work did not lead to positional cloning of P450c1 $\alpha$ , knowledge that the gene responsible for VDDR-I mapped to 12q14 provided powerful evidence that the subsequently cloned human P450c1 $\alpha$  gene was indeed the correct gene, as Fu *et al.* (1997b) mapped the gene for P450c1 $\alpha$  to chromosome 12 by somatic cell hybridization and St-Arnaud *et al.* (1997) mapped it to 12q13.1-q13.3 by fluorescence *in situ* hybridization (FISH). Furthermore, the microsatellite mapping using only 3 markers permitted Wang *et al.* (1998) to trace the ancestry of each identified mutation and show that one common mutation has arisen in multiple independent events.

Several groups have now studied the molecular genetics of P450c1 $\alpha$  in VDDR-I. While our first patient (Fu *et al.*, 1997a) and the subsequent four consanguineous patients of Kitanaka *et al.* (1998) proved that mutations in P450c1 $\alpha$  could cause VDDR-I, it was not yet clear if all patients with the clinical VDDR-I syndrome had the same disease (i.e., vitamin D 1 $\alpha$ -hydroxylase deficiency). Also, although it had been well established that VDDR-I was very common in French Canada (De Braekeleer, 1991), it was not clear if all these patients carried the same mutation; in fact careful linkage analysis of VDDR-I families to microsatellite markers in the 12q13-q14 locus had indicated that the French Canadian VDDR-I population derived from two independent founder effects (Labuda *et al.*, 1996). Finally, there was no structure/function information about the enzyme.

By mid 1999, five reports of the genetics of 1 $\alpha$ -hydroxylase deficiency had appeared (Fu *et al.*, 1997a; Kitanaka *et al.*, 1998; Wang *et al.*, 1998; Yoshida *et al.*, 1998; Smith *et al.*, 1999), describing 32 patients in 28 families, carrying a total of 20 different mutations, including 13 amino acid replacement (mis-sense) mutations (Figure 3.5); more are sure to appear before the publication of this book. All of these patients had typical hormonal findings of VDDR-I, which is now more accurately termed 1 $\alpha$ -hydroxylase deficiency, including hypocalcemia, hypophosphatemia, high serum alkaline phosphatase activity, high serum PTH concentrations, normal serum 25OHD concentrations and low 1,25(OH) $_2$ D concentrations. By far the largest study, reporting more than half of all reported patients to

date, was the study of Wang and colleagues that included 19 patients from 17 families representing multiple ethnic groups (Wang *et al.*, 1998). The clinical and genetic data for these patients are summarized in Table 3.1. All patients had radiographic evidence of rickets and all responded to physiologic replacement doses of  $1,25(\text{OH})_2\text{D}$ . Because the P450c1 $\alpha$  gene is small and its complete sequence had been reported in late 1997 (Fu *et al.*, 1997b), a procedure for PCR-amplification of the whole gene from genomic DNA as a single 4.2kb product was developed (Wang *et al.*, 1998). This DNA was then subjected to direct sequencing on both strands, identifying mutations in all patients. Sequencing the mutant exons from both parents identified the parental origin of all mutations; the inheritance of each mutation was then confirmed by performing microsatellite haplotyping of chromosome 12q13 using the markers D12S90, D12S305 and D12S104 in all patients and parents (Wang *et al.*, 1998).

By examining these same microsatellite markers, Labuda and coworkers had previously shown that the French Canadian patients with VDDR-I carried one of two haplotypes. Those whose ancestry could be traced to the Charlevoix-Seguenay-LacSaint Jean area of Quebec (the “Charlevoix” population) carried haplotype 4–7–1, while those whose ancestry could be traced to the maritime provinces of Eastern Canada (the “Acadian” population) carried the haplotype 6–7–2 (Labuda *et al.*, 1996). Among the Five French Canadian families studied by Wang *et al.* (1998), 9 of 10 unique alleles carried the 4–7–1 haplotype and all nine carried the same mutation, the deletion of a guanosine at position 958 ( $\Delta\text{G}958$ ). This mutation lies in codon 88 (in exon 2), changes the reading frame, and leads to premature translational termination, thus ablating all enzyme activity. Homozygosity for this same mutation was identified in three of four families studied by Yoshida *et al.* (1998). This mutation deletes the G in the sequence 5'ACGT3', which is recognized by the endonucleases *Tai* I and *Mae* II; this feature was used to design a rapid, accurate PCR-based diagnostic tactic to detect this mutation in genomic DNA from any source (Wang *et al.*, 1998) (Figure 3.6). There are several additional *Tai* I and *Mae* II sites in the P450c1 $\alpha$  gene, including 3 just downstream in exon 3. However, PCR amplification of genomic DNA with a sense oligonucleotide corresponding to bases 52–81 and an anti-sense oligonucleotide corresponding to bases 1612–1638 generates a 1458bp fragment that contains only the *Tai* I/*Mae* II site affected by the  $\Delta\text{G}958$  mutation. When unaffected DNA is amplified and digested with *Tai* I or *Mae* II, two fragments of 778 and 680bp are seen; by contrast, DNA carrying the  $\Delta\text{G}958$  mutation will remain undigested as the 1458bp fragment (Figure 3.6).

Among the four French Canadian families studied by Yoshida *et al.*, three were homozygous for the  $\Delta\text{G}958$  mutation and one was homozygous for duplication of a 7bp sequence in exon 8 (Yoshida *et al.*, 1998). Based on the regions of Canada from which each patient originated, Yoshida *et al.* inferred that the  $\Delta\text{G}958$  is the Charlevoix mutation and the 7bp duplication is the Acadian mutation. The combination of mutational and microsatellite analyses by Wang *et al.* (1998) prove that  $\Delta\text{G}958$  is indeed the Charlevoix mutation; however, Yoshida *et al.* (1998) did not perform microsatellite haplotyping. Hence, it is not known whether the patient who was homozygous for the 7bp duplication was “Acadian” as defined by carrying the 6–7–2 haplotype. In fact, Wang *et al.* found the 7bp duplication on seven distinct alleles. Four of these alleles bore a similar but different haplotype, 9–7–2, but were found in three different ethnic groups: Polish, Chinese and Hispanic. The other three alleles carrying the 7bp duplication carried the haplotypes 9–6–2, 9–3–3 and 6–6–1, and

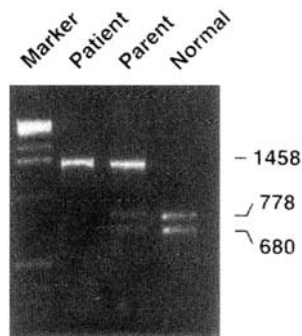
**Table 3.1** Clinical and genetic data in patients with 1 $\alpha$ -hydroxylase deficiency.

<i>Patient</i>	<i>Ethnicity</i>	<i>Ca</i> [8.5–10.5] mg/dl	<i>P</i> [4–6] mg/dl	<i>Alk Phos</i> [150– 250] IU/L	<i>PTH</i> [10–60] pg/ml	<i>25OHD</i> [10–60] ng/ml	<i>1,25(OH)<sub>2</sub>D</i> [20–60] pg/ml	<i>Microsatellite</i> <i>haplotype</i> (Maternal/ Paternal)	<i>Mutations<sup>d</sup></i> (Maternal/ Paternal)
1a <sup>a</sup>		9.6	3.8	275	265 <sup>b</sup>	30	11	4-1-1/9-6-2	T409I/7bpdup
1b <sup>a</sup>	Filipino	9.8	3.8	356	300 <sup>b</sup>	59	16	4-1-1/9-6-2	T409I/7bpdup
2	White-USA	6.2	3.7	1042	171	103	55 <sup>c</sup>	9-5-2/4-7-1	R389H/958ΔG
3	French Canadian	6.5	3.6	625	“high”	37	8	4-7-1/9-1-2	958ΔG/R389H
4	French Canadian	6.1	2.5	1720	980 <sup>b</sup>	30	<2	4-7-1/4-7-1	958ΔG/958ΔG
5a	French Canadian	5.5	4.7	1900	–	–	–	4-7-1/4-7-1	958ΔG/958ΔG
5b	French Canadian	“low nl”	–	600	–	–	–	4-7-1/4-7-1	958ΔG/958ΔG
6 <sup>a</sup>	French Canadian	<8.0	2.6	1300	–	256	<20	4-7-1/4-7-1	958ΔG/958ΔG
7	White-USA	6.1	2.8	1958	2231	40	9	4-7-1/4-7-1	958ΔG/958ΔG
8	Polish	7.4	3.3	920	524 <sup>b</sup>	24	15	6-7-2/4-1-2	P497R/E189L
9	Polish	8.4	2.0	3120	“high”	124	4	9-5-2/9-5-2	W241X/W241X
10	Polish	7.6	5.5	1730	–	304	44	9-7-2/9-7-2	7bpdup/7bpdup
11 <sup>a</sup>	Chinese	5.8	3.0	1005	90	–	<17	9-7-2/9-7-2	7bpdup/Q65H
12	French Canadian	7.2	2.8	1515	101	58	<5	4-7-1/4-7-1	958ΔG/958ΔG
13	White-USA	5.7	4.4	1183	472	45	19	4-1-1/9-3-3	2bpdup/7bpdup
14	Black-USA	8.0	2.9	1113	945 <sup>b</sup>	46	<5	4-5-1/6-6-1	R429P/7bpdup
15	Haitian	6.8	3.8	1660	–	45	<10	4-7-2/4-7-2	R453C/R453C
16	Hispanic	8.1	2.2	3542	862	86	10	4-1-1/9-7-2	212ΔG/7bpdup
17	White-USA	7.4	1.5	1880	377	110	<12	...	1921ΔG,1984Δc <sup>e</sup>

**Note**

- a Had been on treatment since infancy; all medications held for >1 week,
- b C-terminal assays (normal range <330ng/ml).
- c Receiving calcitriol 0.25μg/d.
- d Amino acid replacement mutations are indicated according to codon number; nucleotide deletions are numbered from the transcriptional start site; 7bpdup refers to the insertion of an additional CCCACCC sequence in codons 438–442.
- e Parental alleles unavailable for assignment.

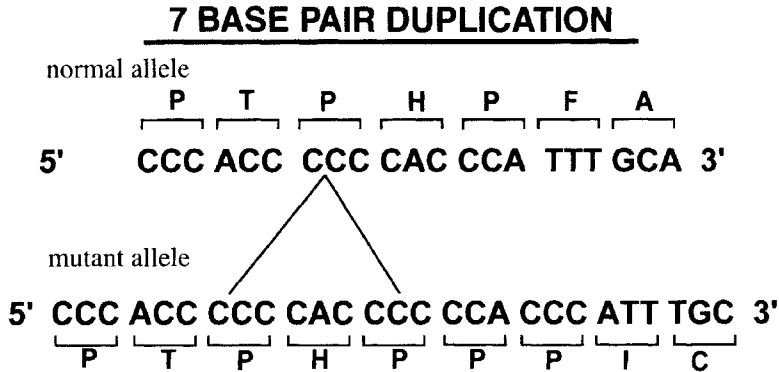
were found among Filipino, Caucasian American and Black American patients (Table 3.1). Thus, the 7bp duplication appears to have arisen *de novo* among different ethnic groups, probably as the result of a slipped-strand mispairing mechanism during meiosis (Figure 3.7) (Levinson and Gutman, 1987). Finally, a Polish patient was heterozygous for the “Acadian” 6–7–2 haplotype, but that allele carried the mis-sense mutation P497R rather than the 7bp duplication (Wang *et al.*, 1998). Thus, the identity of the “Acadian” mutation has not been established; however, it may well be the 7bp duplication as that mutation has arisen in so many different populations.



**Figure 3.6 Genetic diagnosis of the  $\Delta$ G958 mutation commonly found among the Charlevoix French Canadian population.** Genomic DNA samples from a homozygously affected patient, an obligately heterozygous parent, and a homozygously unaffected normal control were amplified as described (Wang *et al.*, 1998), digested with *Tai* I, and run on a gel with molecular size markers. The patient’s DNA, carrying  $\Delta$ G958 on both alleles, cannot be cut; the normal DNA is cut to completion, and half of the heterozygote’s DNA is cut.

### Structure-function correlations in P450c1 $\alpha$

Mis-sense mutations causing clinical disease can provide insight into the structure-function relationships of P450 enzymes, especially when mutations are identified



**Figure 3.7 The 7bp duplication.** Upper line: The sequence CCCACCC is normally duplicated in exon 8, encoding residues 438 to 442 (Pro-Thr-Pro-His-Pro). Lower line: The mutation involves the insertion of a third copy of the CCCACCC sequence, changing the reading frame, beginning with residue 443. The “triplication” is arbitrarily shown as an insert at codon 440 between the two normal copies of the CCCACCC sequence; it is impossible to specify which of the three copies in the mutant sequence is “new”.

that retain partial or selective activities (e.g., ablation of the 17,20-lyase activity of P450c17 (CYP17), while retaining 17 $\alpha$ -hydroxylase activity) (Geller *et al.*, 1997). As of mid 1999, 13 different mis-sense mutations have been described in P450c1 $\alpha$ . Kitanaka and colleagues identified four mis-sense mutations in their four consanguineous Japanese families, and

tested these in a promoter/reporter transactivation assay based on activation of the VDR by  $1,25(\text{OH})_2\text{D}$  (Kitanaka *et al.*, 1998). This assay, which appears to be able to distinguish activity that is  $>5\%$  above background, found no activity in any of the four mutants was observed, consistent with the VDDR-I phenotype of the patients. Wang *et al.* (1998) identified 7 additional mis-sense mutations and expressed the correspondingly mutated cDNAs in transfected MA-10 cells to assess enzymatic activity. In these experiments, the background level of  $1\alpha$ -hydroxylase activity in MA-10 cells was about 65 pg of  $1,25(\text{OH})_2\text{D}$  produced per plate of cells, which is  $\sim 4\%$  of the level

**Table 3.2** Activity of the P450c1 $\alpha$  mutants.

<i>Construct</i>	<i>1,25(OH)<sub>2</sub>D</i> (pg/plate)*
Vector	65
Q65H	68
E189L	85
R389H	45
T409I	52
R429P	58
R435C	65
P497R	60
Wild-type	1,530

Note

\* Detection limit, 8pg/plate.

achieved by expression of the wild-type enzyme in these cells; by contrast, the activities of the seven mutants ranged from 45–85 pg (2.9% to 5.6% of control) (Table 3.2), i.e., levels indistinguishable from the vector control. Smith *et al.* (1999) identified two additional mutations (S323Y and V478G) in a compound heterozygous patient. These authors found  $1\alpha$ -hydroxylase activity in macrophages from normal individuals and from the obligately heterozygous parents, but not in the macrophages from the patient, suggesting that both mutations severely impair  $1\alpha$ -hydroxylase activity. Thus of the 13 mis-sense mutations identified to date, none has resulted in a P450c1 $\alpha$  enzyme with decreased, rather than undetectable activity. This may be because not enough distinct patients have been studied yet; alternatively, it is possible that mutations that decreased, rather than completely eliminated,  $1\alpha$ -hydroxylase activity may not produce a clinical phenotype.

The structures of several bacterial P450 enzymes have been determined by X-ray crystallography, including the class I enzymes CYP101 (Poulos *et al.*, 1986), CYP108 (Hasemann *et al.*, 1994), and CYP107 (Cupp-Vickery and Poulos, 1995), and the class II enzyme CYP102 (Ravichandran *et al.*, 1993). Comparisons of the structures of these enzymes shows remarkable conservation of their topology and tertiary structures, despite low amino acid sequence identity (Wang *et al.*, 1998) (Figure 3.8). This permitted the preliminary assignments of the locations of the seven amino acid replacement mutations they found in P450c1 $\alpha$ . The mutation Q65H is in  $\alpha$ -helix A', T409I is in strand 3 of  $\beta$ -sheet 1, and R389H is in strand 4 of  $\beta$ -sheet 1. Although these mutations are distant from one another in terms of their amino acid numbers, they lie in the clustered  $\beta$ -sheet domain that

P450c1 $\alpha$	MTQTLYASRVFHRVRWAPELGASLGVEYHSA-----RRSLADIPGSPSPFLAELFCCKGGLS	59
P450cam	-----MTTETIQSNANLAPLPPHVPEHLVDFDPMYNPSNLISA-	37
P450terp	-----MDARATIP <b>EH</b> ATITVLTPQGYA-	22
P450eryF	-----MTTVPDLESDFHV-----	14
	$\nabla^{Q53H}$ $\alpha A'$	
P450c1 $\alpha$	RI <b>KE</b> LQVQGAHFQ---PWMLASFG-TV <b>RT</b> VVVAAPALVE <b>KL</b> LROBGRPRER--CS-----FSP <b>TE</b> HRRCRQ <b>RA</b> C	124
P450cam	GVQ <b>RA</b> NVVLQESNV--PDLWTRCNG--GH <b>MI</b> AT <b>RQ</b> L <b>TR</b> EAYEDYRHFSE--CP-----FIP <b>RE</b> AG <b>EA</b> Y-	97
P450terp	-- <b>DD</b> EV <b>IT</b> Y <b>PA</b> FW <b>KL</b> AD <b>EQ</b> PL <b>MA</b> HEGYD <b>PM</b> AT <b>KA</b> D <b>VM</b> Q <b>IG</b> K <b>Q</b> GL <b>ES</b> NA <b>GS</b> E--CP-----LLYD <b>Q</b> NE <b>AF</b> MR <b>IS</b>	92
P450eryF	--- <b>DM</b> Y <b>RT</b> Y <b>AL</b> K <b>RL</b> ET <b>AT</b> EV <b>TF</b> PR <b>FL</b> GD <b>Q</b> -- <b>AW</b> LV <b>GT</b> GY <b>DE</b> KA <b>AL</b> SL <b>DL</b> SS <b>DP</b> K <b>KK</b> Y <b>PG</b> VE <b>VE</b> FP <b>AL</b> YG <b>FP</b> <b>ED</b> V <b>HT</b> FA <b>---</b>	87
	$\alpha A'$ $\beta$ 1-1 $\beta$ 1-2 $\alpha B$ $\beta$ 1-5 $\alpha B$ $\nabla^{E189L}$	
P450c1 $\alpha$	----G <b>LL</b> TA <b>EG</b> -- <b>KE</b> W <b>QR</b> LS <b>EL</b> LA <b>PL</b> LL <b>RP</b> QA <b>AR</b> YAG <b>TI</b> AN <b>V</b> CD <b>LV</b> RR <b>LR</b> Q <b>R</b> GRGT <b>GP</b> PA <b>LV</b> RD <b>VA</b> GE <b>TF</b> PK <b>GL</b> SG <b>IA</b> AV <b>LL</b> G	204
P450cam	-----D <b>FI</b> PT <b>SD</b> MP <b>PE</b> Q <b>RQ</b> FR <b>AL</b> AN <b>Q</b> V <b>VG</b> MP <b>V</b> DK-----L <b>EN</b> RI <b>Q</b> EL <b>AC</b> SL <b>IE</b> SL <b>RP</b> --Q <b>Q</b> C <b>QN</b> FT <b>ED</b> Y <b>AE</b> FP <b>PI</b> RI <b>PK</b> LA <b>GL</b>	170
P450terp	GG <b>CP</b> H <b>VI</b> DS <b>LT</b> SM <b>DP</b> PT <b>AT</b> TA <b>RG</b> L <b>IA</b> W <b>PF</b> AS <b>IR</b> K---L <b>EN</b> IR <b>RI</b> QA <b>AS</b> V <b>RL</b> LD <b>F</b> --D <b>GE</b> CD <b>FM</b> TC <b>DA</b> LT <b>YP</b> LE <b>VV</b> MT <b>AL</b> GV	172
P450eryF	-----T <b>NM</b> GT <b>SD</b> PP <b>TT</b> ET <b>RL</b> K <b>LV</b> S <b>QE</b> PT <b>VR</b> VR <b>AM</b> -----R <b>PR</b> VE <b>Q</b> IT <b>AE</b> LL <b>DE</b> VGD <b>Q</b> --S <b>GV</b> VD <b>IV</b> DR <b>FA</b> HL <b>VE</b> IR <b>VI</b> CK <b>EL</b> GV	159
	$\alpha C$ $\alpha C'$ $\alpha D$ $\beta$ 1-1 $\alpha E'$ $\alpha E$	
P450c1 $\alpha$	SRIG <b>LE</b> AQ <b>VP</b> PD <b>TE</b> TF <b>IR</b> AV <b>GS</b> V <b>VF</b> ST <b>LL</b> TH-----AM <b>PH</b> W <b>LR</b> HL <b>LV</b> PG <b>W</b> GR <b>LCK</b> WD <b>Q</b> MF <b>AF</b> A <b>QR</b> H <b>VE</b> RR	271
P450cam	-----P <b>EE</b> DI <b>PL</b> K <b>Y</b> LT <b>Q</b> MT <b>R</b> -----PD <b>---</b> G <b>SM</b> -----T <b>FA</b> RA <b>K</b> AL <b>Y</b> DT <b>LL</b> PI <b>IE</b> Q <b>RG</b> K	215
P450terp	-----P <b>ED</b> DE <b>FL</b> ML <b>CT</b> Q <b>DF</b> FG <b>V</b> HE <b>PD</b> EQ <b>AV</b> A-----AP <b>RQ</b> SA <b>DE</b> AA <b>R</b> --R <b>PE</b> ET <b>AT</b> TT <b>DT</b> Y <b>PG</b> PT <b>VD</b> RR <b>SC</b>	233
P450eryF	-----D <b>E</b> KY <b>R</b> GE <b>PF</b> GR <b>WS</b> SE <b>LV</b> -----MD <b>PE</b> RA <b>E</b> -----Q <b>RQ</b> QA <b>R</b> V <b>Y</b> VF <b>IL</b> DL <b>VE</b> RR <b>TE</b>	206
	$\alpha F$ $\alpha G$	
P450c1 $\alpha$	K <b>AA</b> AM <b>R</b> NG <b>Q</b> PE <b>KD</b> LE <b>SG</b> A <b>HLT</b> --H <b>FL</b> FR--E <b>EL</b> PA <b>Q</b> S <b>IL</b> GN <b>VT</b> ELL <b>AG</b> VD <b>TV</b> ST <b>LS</b> MA <b>LY</b> EL <b>SR</b> HP <b>EQ</b> TA <b>LE</b> SE <b>IT</b> AA <b>LS</b> PG <b>SS</b>	356
P450cam	PG <b>TD</b> AI <b>IS</b> IV <b>AN</b> -----G <b>Q</b> V--N <b>GR</b> --P <b>IT</b> SD <b>EAK</b> MC <b>GL</b> LL <b>VG</b> GL <b>TV</b> V <b>ML</b> SP <b>ME</b> FL <b>AK</b> SP <b>ER</b> Q <b>EL</b> IER <b>P</b> -----	279
P450terp	PK <b>DD</b> W <b>MS</b> LL <b>AN</b> -----S <b>K</b> L--D <b>GN</b> --Y <b>IDD</b> KY <b>IN</b> AT <b>Y</b> VA <b>IT</b> AG <b>HD</b> TT <b>SS</b> SG <b>AA</b> IT <b>GL</b> SR <b>NP</b> Q <b>EL</b> AK <b>SD</b> P-----	297
P450eryF	PG <b>DD</b> LL <b>S</b> AL <b>IR</b> V <b>Q</b> -----D <b>---</b> DD <b>D</b> GR <b>L</b> SA <b>DEL</b> TS <b>IA</b> LV <b>LL</b> AG <b>PE</b> AS <b>VL</b> IG <b>IT</b> GT <b>LL</b> TH <b>EP</b> D <b>Q</b> AL <b>VR</b> ED <b>P</b> -----	271
	$\alpha H$ $\beta$ 5-1 $\beta$ 5-2 $\alpha I$ $\nabla^{R429P}$ $\alpha I$ $\nabla^{R429P}$	
P450c1 $\alpha$	A <b>Y</b> PS <b>AT</b> VL <b>SQ</b> PL <b>KL</b> K <b>AV</b> W <b>KE</b> VL <b>RL</b> Y <b>VP</b> V <b>GN</b> SR--V <b>PD</b> KD <b>IH</b> VGD <b>Y</b> I <b>IP</b> KN <b>TL</b> VL <b>TH</b> CA <b>Y</b> TSR <b>DP</b> QA <b>FP</b> EP <b>NS</b> FR <b>PA</b> RW <b>LG</b> E <b>P</b> -----T	439
P450cam	-----E <b>RI</b> PA <b>ACK</b> EL <b>LR</b> AF <b>SL</b> V--AD <b>CR</b> IL <b>TS</b> D <b>Y</b> E <b>PH</b> G--V <b>Q</b> L <b>K</b> GD <b>Q</b> ILL <b>PK</b> SL <b>GL</b> SD <b>ER</b> NA <b>CP</b> M <b>VD</b> FS <b>RQ</b> K <b>VS</b> -----	347
P450terp	-----A <b>L</b> IP <b>KL</b> VD <b>AV</b> Y <b>DT</b> AP <b>V</b> KS <b>FM</b> TA <b>LAD</b> TE <b>VR</b> G--Q <b>NI</b> K <b>R</b> GD <b>RI</b> ML <b>ST</b> PS <b>AM</b> DE <b>EV</b> FN <b>PD</b> EF <b>DI</b> TR <b>FP</b> NR-----	366
P450eryF	-----S <b>AL</b> PN <b>AV</b> EL <b>IL</b> RY <b>IA</b> PP <b>ET</b> TR <b>FA</b> AE <b>VE</b> IR <b>G</b> --V <b>AI</b> PO <b>Y</b> ST <b>VL</b> V <b>ANG</b> AAN <b>DR</b> PK <b>Q</b> FP <b>D</b> PH <b>R</b> FD <b>VT</b> TR <b>TR</b> G-----	340
	$\alpha K$ $\beta$ 1-4 $\beta$ 2-1 $\beta$ 2-2 $\beta$ 1-3 $\alpha K'$ Meander	
	$\nabla^{R453C}$ $\nabla^{P479R}$	
P450c1 $\alpha$	PH <b>PF</b> AS <b>LP</b> FG <b>PK</b> RS <b>CM</b> OR <b>EL</b> LA <b>EL</b> EL <b>Q</b> MA <b>LQ</b> IL <b>TH</b> FE <b>V</b> --Q <b>PE</b> PG--A <b>AP</b> VR--PK <b>TR</b> TV <b>LV</b> PS <b>ER</b> IN <b>LQ</b> FL <b>DR</b> ---	508
P450cam	-----H <b>TF</b> FG <b>H</b> SG <b>HL</b> CL <b>Q</b> EL <b>AR</b> RE <b>IV</b> TL <b>KE</b> W <b>LT</b> RI <b>PD</b> FS <b>IA</b> PG <b>A</b> --Q <b>I</b> Q <b>HK</b> --S <b>G</b> IVSG <b>VQ</b> AL <b>PL</b> W <b>ND</b> PA <b>TK</b> AV	414
P450terp	-----H <b>LG</b> FG <b>W</b> GA <b>HM</b> CL <b>Q</b> EL <b>AK</b> LE <b>KI</b> FF <b>KE</b> LL <b>PK</b> L--S <b>VEL</b> SG--P <b>RL</b> V--A <b>TF</b> NV <b>GG</b> PK <b>NV</b> IR <b>PT</b> KA-----	428
P450eryF	-----H <b>LS</b> EG <b>Q</b> GI <b>HF</b> CM <b>RL</b> AK <b>LE</b> GE <b>VA</b> LR <b>AL</b> GR <b>VF</b> PA <b>LS</b> IG <b>DA</b> DD <b>VV</b> WR <b>RS</b> --L <b>LR</b> RG <b>DI</b> HL <b>PL</b> VR <b>LD</b> G-----	404
	Cys-pocket $\alpha L$ $\beta$ 3-3 $\beta$ 4-1 $\beta$ 4-2 $\beta$ 3-2	

Figure 3.8 Alignment of the amino acid sequence of human P450c1 $\alpha$  (CYP27B1) (top) with the sequences of the crystallographically-solved bacterial class I P450 proteins P450cam (CYP101), P450terp (CYP108) and P450eryF (CYP107). The names of the various  $\alpha$ -helices and  $\beta$ -sheets are given below, with the  $\alpha$ -helices highlighted in boldface and the  $\beta$ -sheets underlined. The locations of the “meander” and the Cys-pocket are also shown. The locations of the amino acid replacement mutations identified in the P450c1 $\alpha$  are shown above its sequence with downward-pointing arrows.

probably interacts with the inner mitochondrial membrane and may define the substrate entry channel. While it is not yet possible to define the exact lesion caused by each of these mutations, their location suggests they are likely to be confrontational mutants that disrupt the ability of the enzyme to bind substrate, rather than disrupting the catalytic site or the redox-partner binding site. The mutant E189L lies in the E-helix and would disrupt the four-helix bundle consisting of the D, E, I and L helices, and thus would cause a significant disruption in the P450 structure. The mutant R429P inserts a proline at the junction of the K' helix and the meander, changing the direction of the carbon backbone and grossly disrupting the meander. The mutant R453C, which is only two residues away from the thiolate cysteine 455, disrupts a salt bridge that interacts with the heme propionate, very similarly to the corresponding R440C mutation of P450c17 that causes complete 17 $\alpha$ -hydroxylase deficiency (Fardella *et al.*, 1994) and the R435C mutation in CYP19 that causes complete aromatase deficiency (Conte *et al.*, 1994). Finally, the P479R mutant lies near strand 3 of  $\beta$ -

sheet 3, which participates in defining the top of the substrate-binding pocket; the directional change in the  $\alpha$ -carbon backbone resulting from insertion of a proline probably disrupts substrate binding. Thus these P450c1 $\alpha$  mis-sense mutations lie in locations predicted to disrupt enzymatic activity, consistent with the demonstration that each lacks detectable 1 $\alpha$ -hydroxylase activity.

## REFERENCES

- Akeno, N., Saikatsu, S., Kawane, T. and Horiuchi, N. (1997) Mouse vitamin D-24-hydroxylase: molecular cloning, tissue distribution, and transcriptional regulation by 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub>. *Endocrinology* **138**, 2233–2240.
- Armbricht, H.J. and Boltz, M.A. (1991) Expression of 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 in kidney and intestine. Effect of 1,25-dihydroxyvitamin D and age. *FEBS Lett.* **292**, 17–20.
- Armbricht, H.J., Okuda, K., Wongsurawat, N., Nemani, R.K., Chen, M.L. and Boltz, M.A. (1992) Characterization and regulation of the vitamin D hydroxylases. *J. Steroid Biochem. Molec. Biol.* **43**, 1073–1081.
- Armbricht, H.J., Wongsurawat, N., Zenser, T.V. and Davis, B.B. (1982) Differential effects of parathyroid hormone on the renal 1,25-dihydroxyvitamin D<sub>3</sub> and 24,25-dihydroxyvitamin D<sub>3</sub> production of young and adult rats. *Endocrinology* **111**, 1339–1344.
- Andersson, S., Davis, D., Dahlback, H., Jornvall, H. and Russell, D. (1989) Cloning structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264**, 8222–8229.
- Auchus, R.J., Lee, T.C. and Miller, W.L. (1998) Cytochrome b<sub>5</sub> augments the 17,20 lyase activity of human P450c17 without direct electron transfer. *J. Biol. Chem.* **273**, 3158–3165.
- Baxter, L.A. and DeLuca, H.F. (1976) Stimulation of 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase by phosphate depletion. *J. Biol. Chem.* **251**, 3158–3161.
- Bell, N.H., Shaw, S. and Turner, R.T. (1984) Evidence that 1,25-dihydroxyvitamin D<sub>3</sub> inhibits the hepatic production of 25-hydroxyvitamin D in man. *J. Clin. Invest.* **74**, 1540–1544.
- Beringer, V.M., Shany, S., Alkalay, D., Beringer, J., Dekel, S., Salen, G., Tint, G.S. and Gazit, D. (1992) Osteoporosis, bone fractures, and low vitamin D in cerebrotendinous xanthomatosis. *Metab. Clin. Exp.* **42**, 69–74.
- Bhattacharyya, M.H. and DeLuca, H.F. (1974) Subcellular location of rat liver calciferol-25-hydroxylase. *Arch. Biochem. Biophys.* **160**, 58–62.
- Bikle, D.D., Nemanic, M.K., Gee, E. and Elias, P. (1986a) 1,25-dihydroxyvitamin D<sub>3</sub> production by human keratinocytes. *J. Clin. Invest.* **78**, 557–566.
- Bikle, D., Nemanic, M., Whitney, J. and Elias, P. (1986b) Neonatal human foreskin keratinocytes produce 1,25-dihydroxyvitamin D<sub>3</sub>. *Biochemistry* **25**, 1545–1548.
- Bikle, D. and Pillai, S. (1993) Vitamin D, calcium and epidermal differentiation. *Endocr. Rev.* **14**, 3–19.
- Bjorkhem, I. and Holmberg, I. (1978) Assay and properties of a mitochondrial 25-hydroxylase active on vitamin D<sub>3</sub>. *J. Biol. Chem.* **253**, 842–849.
- Black, S.D. and Coon, M.J. (1987) P-450 cytochromes: structure and function. *Adv. Enzymol. Relat. Areas Mol. Biol.* **60**, 35–87.
- Booth, B.E., Tsai, H.C. and Morris, Jr., R.C. (1985) Vitamin D status regulates 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase and its responsiveness to parathyroid hormone in the chick. *J. Clin. Invest.* **75**, 155–161.

- Brentano, S.T. and Miller, W.L. (1992) Regulation of human P450<sub>scc</sub> and adrenodoxin mRNAs in JEG-3 cytotrophoblast cells. *Endocrinology* **131**, 3010–3018.
- Brenza, H., Kimmel-Jehan, C., Jehan, F., Shinki, T., Wakino, S., Hideharu, A., Suda, T. and DeLuca, H. (1998) Parathyroid hormone activation of the 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase gene promoter. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1387–1391.
- Breslau, N.A. (1988) Normal and abnormal regulation of 1,25(OH)<sub>2</sub>D Synthesis. *Am. J. Med. Sci.* **296**, 417–425.
- Cali, J.J. and Russell, D.W. (1991) Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidation reaction in bile acid biosynthesis. *J. Biol. Chem.* **266**, 7774–7778.
- Casella, S.J., Reiner, B.J., Chen, T.C., Holick, M.F. and Harrison, H.E. (1994) A possible genetic defect in 25-hydroxylation as a cause of rickets. *J. Pediat.* **124**, 929–932.
- Chang, C.-Y., Wu, D.-A., Lai, C.-C., Miller, W.L. and Chung, B. (1988) Cloning and structure of the human adrenodoxin gene. *DNA* **7**, 609–615.
- Chen, K.S. and DeLuca, H.F. (1995) Cloning of the human 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-24-hydroxylase gene promoter and identification of two vitamin D-responsive elements. *Biochim. Biophys. Acta.* **1263**, 1–9.
- Chen, K.S., Prahl, J.M. and DeLuca, H.F. (1993a) Isolation and expression of human 1,25-dihydroxyvitamin D<sub>3</sub> 24-hydroxylase cDNA. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4543–4547.
- Chen, M.L., Boltz, M.A. and Armbrecht, H.J. (1993b) Effects of 1,25-dihydroxyvitamin D<sub>3</sub> and phorbol ester on 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase cytochrome P450 messenger ribonucleic acid levels in primary cultures of rat renal cells. *Endocrinology* **132**, 1782–1788.
- Chung, B., Matteson, K.J., Voutilainen, R., Mohandas, T.K. and Miller, W.L. (1986) Human cholesterol side-chain cleavage enzyme, P450<sub>scc</sub>: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8962–8966.
- Conte, F.A., Grumbach, M.M., Ito, Y., Fisher, C.R. and Simpson, E.R. (1994). A syndrome of female pseudoherniaphrodis, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450<sub>arom</sub>). *J. Clin. Endocrinol. Metab.* **78**, 1287.
- Cupp-Vickery, J.R. and Poulos, T.L. (1995) Structure of cytochrome P450<sub>eryF</sub> involved in erythromycin biosynthesis. *Nat. Struct. Biol.* **2**, 144–153.
- Dahlback, H. and Wikvall, K. (1988) 25-Hydroxylation of vitamin D by a cytochrome P-450 from rabbit liver mitochondria. *J. Biochem.* **252**, 207–213.
- DeBernardes-Clark, E. and Georgiou, G. (1991) Inclusion bodies and recovery of proteins from the aggregated state, in protein refolding. *ACS symposium series 470*, Washington, DC: p. 1.
- De Braekeleer, M. (1991) Hereditary disorders in Saguenay-Lac-St-Jean (Quebec, Canada). *Hum. Hered.* **41**, 141–146.
- Fardella, C.E., Hum, D.W., Homoki, J. and Miller, W.L. (1994) Point mutation Arg440 to His in cytochrome P450<sub>c</sub> 17 causes severe 17 $\alpha$ -hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **79**, 160–164.
- Fardella, C.E., Hum, D.W., Rodriguez, H., Zhang, G., Barry, F., Bloch, C.A. and Miller, W.L. (1996) Gene conversion in the CYP11B2 gene encoding aldosterone synthase (P450<sub>c11AS</sub>) is associated with, but does not cause, the syndrome of corticosterone methyl oxidase II deficiency. *J. Clin. Endocrinol. Metab.* **81**, 321–326.
- Fardella, C.E. and Miller, W.L. (1996) Molecular biology of mineralocorticoid metabolism. *Annu. Rev. Nutr.* **16**, 443–470.
- Feldman, D., Malloy, P.J. and Gross, C. (1996) Vitamin D: metabolism and action. In: Marcus, R., Feldman, D. and Kelsey, J.S. (eds) *Osteoporosis*. Academic Press, San Diego, pp. 205–235.



- Fraser, D., Kooh, S.W., Kind, H.P., Holick, M.F., Tanaka, Y. and DeLuca, H.F. (1973) Pathogenesis of hereditary vitamin-D-dependent rickets. An inborn error vitamin D metabolism involving defective conversion of 25-hydroxyvitamin D to 1 $\alpha$ ,25-dihydroxyvitamin D.N. *Engl. J. Med.* **289**, 817–722.
- Fraser, D.R. (1980) Regulation of the metabolism of vitamin D. *Physiol. Rev.* **60**, 551–613.
- Fu, G.K., Lin, D., Zhang, M.Y.H., Bikle, D.D., Shackleton, C.H.L., Miller, W.L. and Portale, A.A. (1997a) Cloning of human 25-hydroxy vitamin D-1 $\alpha$ -hydroxylase and mutations causing vitamin D-dependant rickets type J. *Mol. Endocrinol.* **11**, 1961–1970.
- Fu, G.K., Portale, A.A. and Miller, W.L. (1997b) Complete structure of the human gene for the vitamin D 1 $\alpha$ -hydroxylase, P450c1 $\alpha$ . *DNA Cell. Biol.* **16**, 1499–1507.
- Garuti, R., Lelli, N., Barozzini, M., Dotti, M.T., Frederico, A., Bertolini, S. and Calandra, S. (1996) Partial deletion of the gene encoding sterol 27-hydroxylase in a subject with cerebrotendinous xanthomatosis. *J. Lipid Res.* **37**, 662–672.
- Gascon-Barrae, M. and Gamache, M. (1991) Contribution of the biliary pathway to the homeostasis of vitamin D<sub>3</sub> and of 1,25-dihydroxyvitamin D<sub>3</sub>. *Endocrinology* **129**, 2335–2344.
- Geller, D.H., Auchus, R.J., Mendonca, B.B. and Miller, W.L. (1997) The genetic and functional basis of isolated 17,20-lyase deficiency. *Nat. Genet.* **17**, 201–205.
- Geller, D.H., Auchus, R.J. and Miller, W.L. (1999) P450c17 mutations R347H and R358Q selectively disrupt 17,20-lyase activity by disrupting interactions with P450 oxidoreductase and cytochrome b<sub>5</sub>. *Mol. Endocrinol.* **13**, 167–175.
- Gray, R.W. (1981) Control of plasma 1,25-(OH)<sub>2</sub>-vitamin D concentrations by calcium and phosphorus in the rat: effects of hypophysectomy. *Calc. Tiss. Int.* **33**, 485–488.
- Gray, R.W. and Garthwaite, T.L. (1985) Activation of renal 1,25-dihydroxyvitamin D<sub>3</sub> synthesis by phosphate deprivation: evidence for a role for growth hormone. *Endocrinology* **116**, 189–193.
- Gray, R.W., Wilz, D.R., Caldas, A.E. and Lemann, Jr., J. (1977) The importance of phosphate in regulating plasma 1,25-(OH)<sub>2</sub>-vitamin D levels in humans: studies in healthy subjects in calcium-stone formers and in patients with primary hyperparathyroidism. *J. Clin. Endocrinol. Metab.* **45**, 299–306.
- Halloran, B.P., Bikle, D.D., Levens, M.J., Castro, M.E., Globus, R.K. and Holton, E. (1986) Chronic 1,25-dihydroxyvitamin D<sub>3</sub> administration in the rat reduces the serum concentration of 25-hydroxyvitamin D by increasing metabolic clearance rate. *J. Clin. Invest.* **78**, 622–628.
- Halloran, B.P. and Castro, M.E. (1989) Vitamin D kinetics in vivo: effect of 1,25-dihydroxyvitamin D administration. *Am. J. Physiol.* **256**, E686–E691.
- Halloran, B.P. and Spencer, E.M. (1988) Dietary phosphorus and 1,25-dihydroxyvitamin D metabolism: influence of insulin-like growth factor I. *Endocrinology* **123**, 1225–1229.
- Hanukoglu, I., Suh, B.S. and Himmelhoch, S. and Amsterdam, A. (1990) Induction and mitochondrial localization of cytochrome P450<sub>scc</sub> system enzymes in normal and transformed ovarian granulosa cells. *J. Cell Biol.* **111**, 1373–1381.
- Hasemann, C.A., Ravichandran, K.G., Peterson, J.A. and Deisenhofer, J. (1994) Crystal structure and refinement of cytochrome P450<sub>terp</sub> at 2.3 Å resolution. *J. Mol. Biol.* **236**, 1169–1185.
- Henry, H.L. (1986) Influence of a tumor promoting phorbol ester on the metabolism of 25-hydroxyvitamin D<sub>3</sub>. *Biochem. Biophys. Res. Commun.* **139**, 495–500.
- Holick, M.F., MacLaughlin, J.A. and Doppelt, S.H. (1981) Regulation of cutaneous previtamin D<sub>3</sub> photosynthesis in man: skin pigment is not an essential regulator. *Science* **211**, 590–593.
- Hughes, M.R., Brumbaugh, P.P., Hussler, M.R., Wergedal, J.E. and Baylink, D.J. (1975) Regulation of serum 1- $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> by calcium and phosphate in the rat. *Science* **190**, 578–580.

- Janulis, M., Wong, M.S. and Favus, M.J. (1993) Structure-function requirements of parathyroid hormone for stimulation of 1,25-dihydroxyvitamin D<sub>3</sub> production by rat renal proximal tubules. *Endocrinology* **133**, 713–719.
- Kitanaka, S., Takeyama, K., Murayama, A., Sato, T., Okumura, K., Nogami, M., Hasegawa, Y., Niimi, H., Yanagisawa, J., Tanaka, T. and Kato, S. (1998) Inactivating mutations in the 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase gene in patients with pseudovitamin D-deficiency rickets. *N. Engl. J. Med.* **338**, 653–661.
- Kong, X.F., Zhu, X.H., Pei, Y.L., Jackson, D.M. and Holick, M.F. (1999) Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase gene. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6988–6993.
- Kumar, R. (1984) Metabolism of 1,25-dihydroxy vitamin D<sub>3</sub>. *Physiol. Rev.* **64**, 478–504.
- Labuda, M., Labuda, D., Korab-Laskowska, M., Cole, D.E.C., Zietkiewicz, E., Weissenbach, J., Popowska, E., Pronicka, E., Root, A.W. and Glorieux, F.H. (1996) Linkage disequilibrium analysis in young populations: pseudo-vitamin D-deficiency rickets and the founder effect in French Canadians. *Am. J. Hum. Genet.* **59**, 633–643.
- Labuda, M., Morgan, K. and Glorieux, F.H. (1990) Mapping autosomal recessive vitamin D dependency type 1 to chromosomal 12q14 by linkage analysis. *Am. J. Hum. Genet.* **47**, 28–36.
- Leitersdorf, E., Reshef, A., Meiner, V., Levitzki, R., Schwartz, S.P., Dann, E.J., Berkman, N., Cali, J.J., Klapholz, L. and Berginer, V.M. (1993) Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in Jews or Moroccan origin. *J. Clin. Invest.* **91**, 2488–2496.
- Levinson, G. and Gutman, G. (1987) Slipped-strand mispairings: a major mechanism for DNA sequence evolution. *Mol. Biol. Evol.* **4**, 203–221.
- Lin, D., Shi, Y. and Miller, W.L. (1990) Cloning and sequence of the human adrenodoxin reductase gene. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8516–8520.
- Maierhofer, W.J., Gray, R.W. and Lemann, J.Jr. (1984) Phosphate deprivation increases serum 1, 25-(OH)<sub>2</sub>-vitamin D concentrations in healthy men. *Kidney Int.* **25**, 571–575.
- Masumoto, O., Ohyama, Y. and Okuda, K. (1988) Purification and characterization of vitamin D 25-hydroxylase from rat liver mitochondria. *J. Biol. Chem.* **263**, 14256–14260.
- Monkawa, T., Yoshida, T., Wakino, S., Shinki, T., Anazawa, H., DeLuca, H.F., Suda, T., Hayashi, M. and Saruta, T. (1997) Molecular cloning of cDNA and genomic DNA for human 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase. *Biochem. Biophys. Res. Commun.* **239**, 527–533.
- Mornet, E., Dupont, J., Vitek, A. and White, P.C. (1989) Characterization of two genes encoding human steroid 11 $\beta$ -hydroxylase (P45011B). *J. Biol. Chem.* **264**, 20961–20967.
- Morohashi, K., Yoshioka, H., Gotoh, O., Okada, Y., Yamamoto, K., Miyata, T., Sogawa, K. and FujiiKuriyama, Y. and Omura, T. (1987) Molecular cloning and nucleotide sequence of DNA of mitochondrial P-450 (11) of bovine adrenal cortex. *J. Biochem.* **102**, 559–568.
- Murayama, A., Takeyama, K., Kitanaka, S., Kodera, Y., Hosoya, T. and Kato, S. (1998) Inactivating mutations in the 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. *Biochem. Biophys. Res. Comm.* **249**, 11–16.
- Nebert, D.W. and Gonzalez, F.J. (1987) P450 genes: structure, evolution and regulation. *Annu. Rev. Biochem.* **56**, 945–993.
- Ohyama, Y., Noshior, M., Eggertsen, G., Gotoh, O., Kato, Y., Bjorkhem, I. and Okuda, K. (1993) Structural characterization of the gene encoding rat 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase. *Biochemistry* **32**, 76–82.
- Ohyama, Y., Noshiro, M. and Okuda, K. (1991) Cloning and expression of cDNA encoding 25-hydroxyvitamin D<sub>3</sub> 24-hydroxylase. *FEBS Lett.* **278**, 195–198.

- Picado-Leonard, J., Voutilainen, R., Kao, L., Chung, B., Strauss, III, J.F. and Miller, W.L. (1988) Human adrenodoxin: cloning of three cDNAs and cycloheximide enhancement in JEG-3 cells. *J. Biol. Chem.* **263**, 3240–3244, corrected 11016.
- Portale, A.A., Halloran, B.P. and Morris, Jr., R.C. (1987) Dietary intake of phosphorus modulates the circadian rhythm in serum concentration of phosphorus. Implications for the renal production of 1,25-dihydroxyvitamin D. *J. Clin. Invest.* **80**, 1147–1154.
- Portale, A.A., Halloran, B.P. and Morris, Jr., R.C. (1989) Physiologic regulation of the serum concentration of 1,25-dihydroxyvitamin D by phosphorus in normal men. *J. Clin. Invest.* **83**, 1494–1499.
- Portale, A.A., Halloran, B.P., Murphy, M.M. and Morris, Jr., R.C. (1986) Oral intake of phosphorus can determine the serum concentration of 1,25-dihydroxyvitamin D by determining its production rate in humans. *J. Clin. Invest.* **77**, 7–12.
- Portale, A.A., Zhang, M.Y., Hoag, H.M. and Tenenhouse, H.S. (1999) The effect of dietary and serum phosphorus on renal 25-hydroxyvitamin D-1 $\alpha$ - and 24-hydroxylase gene expression. *Bone* **23**, S364.
- Postlind, H., Axen, E., Bergman, T. and Wikvall, K. (1997) Cloning, structure and expression of a cDNA encoding vitamin D<sub>3</sub> 25-hydroxylase. *Biochem. Biophys. Res. Commun.* **241**, 491–497.
- Poulos, T.L., Finzel, B.C. and Howard, A.J. (1986) Crystal structure of substrate-free *Pseudomonas putida* cytochrome P-450. *Biochemistry* **25**, 5314–5322.
- Poulos, T.L., Finzel, B.C. and Howard, A.J. (1987) High-resolution crystal structure of cytochrome P450cam. *J. Mol. Biol.* **195**, 687–700.
- Privalle, C.T., Crivello, J.F. and Jefcoate, C.R. (1983) Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 702–706.
- Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J. (1993) Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* **261**, 731–736.
- Reichel, H., Koeffler, H.P. and Norman, A.W. (1989) The role of the vitamin D endocrine system in health and disease [see comments]. *N. Engl. J. Med.* **320**, 980–991.
- Roy, S. and Tenenhouse, H.S. (1996) Transcriptional regulation and renal localization of 1,25-dihydroxyvitamin D<sub>3</sub>-24-hydroxylase gene expression: effects of the Hyp mutation and 1,25-dihydroxyvitamin D<sub>3</sub>. *Endocrinology* **137**, 2938–2946.
- St-Arnaud, R., Messerlian, S., Moir, J.M., Omdahl, J.L. and Glorieux, F.H. (1997) The 25-hydroxyvitamin D 1- $\alpha$ -hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. *J. Bone Miner. Res.* **12**, 1552–1559.
- Shayiq, R. and Avadhani, N. (1989) Purification and characterization of a hepatic mitochondrial cytochrome P450 active in aflatoxin B1 metabolism. *Biochemistry* **28**, 7546–7554.
- Shinki, T., Jin, C.H., Nishimura, A., Nagai, Y., Ohyama, Y., Noshiro, M., Okuda, K. and Suda, T. (1992) Parathyroid hormone inhibits 25-hydroxyvitamin D<sub>3</sub>-24-hydroxylase mRNA expression stimulated by 1 $\alpha$ ,25-dihydroxy vitamin D<sub>3</sub> in rat kidney but not in intestine. *J. Biol. Chem.* **267**, 13757–13762.
- Shinki, T., Shimada, H., Wakino, S., Anazawa, H., Hayashi, M., Saruta, T. and DeLuca, H. (1997) Cloning and expression of rat 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase cDNA. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12920–12925.
- Shinki, T., Shimada, H., Wakino, S., Shinki, T., Anazawa, H., DeLuca, H.F., Suda, T., Hayashi, M. and Saruta, T. (1997b) Molecular cloning of cDNA and genomic DNA from human 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase. *Biochem. Biophys. Res. Commun.* **239**, 527–533.

- Smith, S.J., Rucka, A.K., Berry, J.L., Davies, M., Mylchreest, S., Paterson, C.R., Heath, D.A., Tassagehji, M., Read A.P., Mee, A.P. and Mawer, E.B. (1999) Novel mutations in the 1 $\alpha$ -hydroxylase (P450c1) gene in three families with pseudovitamin D—deficiency rickets resulting in loss of functional enzyme activity in blood-derived macrophages. *J. Bone Mineral Res.* **14**, 730–739.
- Solish, S.B., Picado-Leonard, J., Morel, Y., Kuhn, R.W., Mohandas, T.K., Hanukoglu, I. and Miller, W.L. (1988) Human adrenodoxin reductase: Two mRNAs encoded by a single gene of chromosome 17cen→q25 are expressed in steroidogenic tissues. *Proc. Natl. Acad. Sci. U.S.A.* **71**, 7104–7108.
- Strewler, G.J. and Rosenblatt, M. (1995) Mineral metabolism. In: Felig, P., Baxter, J.D. and Frohman, L.A. (eds) *Endocrinology and Metabolism*. McGraw-Hill, New York, pp. 1407–1516.
- Su, P., Rennert, H., Shaiqi, R.M., Yamamoto, R., Zheng, Y., Addya, S., Strauss, III, J.F. and Avadhani, N.G. (1990) A cDNA encoding a rat mitochondrial cytochrome P450 catalyzing both the 26-hydroxylations of cholesterol and 25-hydroxylation of Vitamin D<sup>3</sup>: gonadotropic regulation of the cognate mRNA in ovaries. *DNA Cell. Biol.* **9**, 657–665.
- Takayama, K., Kitanaka, S., Sato, T., Kobori, M., Yanagisawa, J. and Kato, S. (1997) 25-Hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase and vitamin D synthesis. *Science* **277**, 1827–1830.
- Tanaka, Y. and Deluca, H.F. (1973) The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. *Arch. Biochem. Biophys.* **154**, 566–574.
- Usui, E., Noshiro, M. and Okuda, K. (1990) Molecular cloning of cDNA for vitamin D<sub>3</sub> 25-hydroxylase from rat liver mitochondria. *FEBS Lett.* **262**, 135–138.
- Wang, J., Lin, C.J., BurrIDGE, S.M., Fu, G.K., Labuda, M., Portale, A.A. and Miller, W.L. (1998) Genetics of vitamin D 1 $\alpha$ -hydroxylase deficiency in 17 families. *Am. J. Hum. Genet.* **63**, 1694–1702.
- Wang, X.M., Zhang, M.Y.H., Miller, W.L. and Portale, A.A. (2002) Novel mutations in patients with 1 $\alpha$ -hydroxylase deficiency that confer partial enzyme activity in vitro. *J. Clin. Endocrinol. Metab.* (In press).
- White, P.C., Curnow, K.M. and Pascoe, L. (1994) Disorders of steroid 11 $\beta$ -hydroxylase isozymes. *Endocr. Rev.* **15**, 421–438.
- Wikvall, K. (1984) Hydroxylations in biosynthesis of bile acids: Isolation of a cytochrome P-450 from rabbit liver mitochondria catalyzing 26-hydroxylation of C-27 steroids. *J. Biol. Chem.* **259**, 3800–3804.
- Yoshida, T., Monkawa, T., Tenenhouse, H., Goodyer, P., Shinki, T., *et al.* (1998) Two novel 1 $\alpha$ -hydroxylase mutations in French-Canadians with vitamin D dependency rickets type I. *Kidney Int.* **54**, 1437–1443.
- Zhang, M.X.H., Wang, X.M., Wang, J.T., Compagnone, N., Mellon, S.H., Olson, J., Tennenhouse, S., Miller, W.L. and Portale, A.A. (2002) Dietary phosphorus transcriptionally regulates 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase gene expression in the proximal renal tubule. *Endocrinology* **143**, 587–595.

# 4.

## BILE ACID BIOSYNTHESIS

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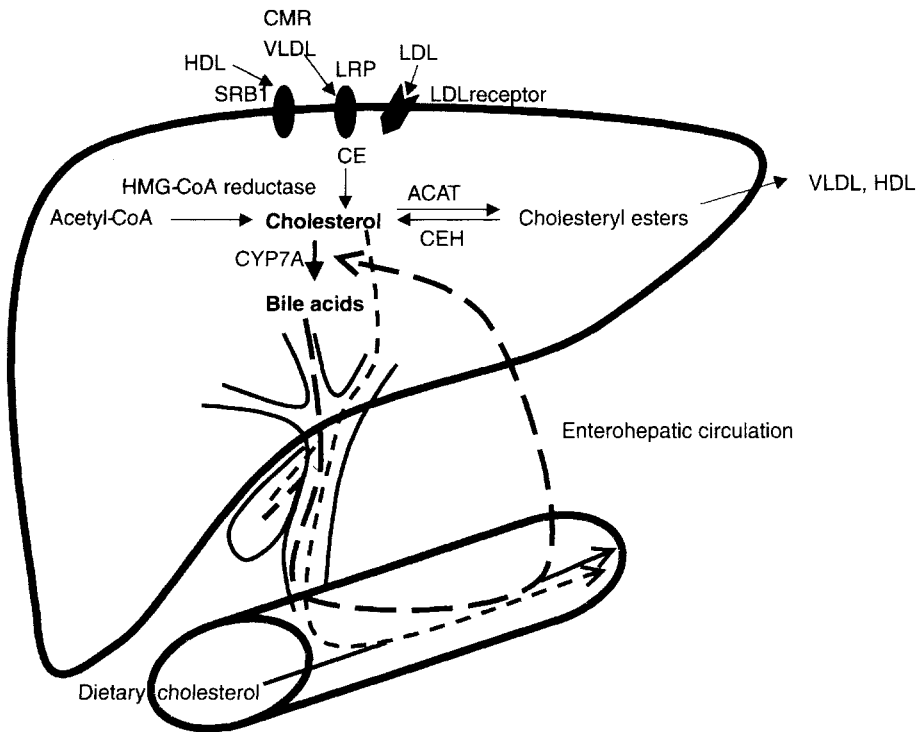
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Conversion of cholesterol to bile acids in the liver is the major catabolic pathway for disposal of cholesterol in mammals. Bile acids are important physiological agents, which facilitates the disposal of sterols and xenobiotics, and absorption and transport of lipid soluble vitamins and fats. Enterohepatic circulation of bile acids is very efficient and plays important physiological roles not only in secretion and distribution of nutrients but also in regulation, the rate of bile acid biosynthesis. Two major bile acid biosynthesis pathways have been established. The classical (or neutral) pathway is initiated by the rate-limiting enzyme, microsomal cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), to synthesize cholic acid (CA) and chenodeoxycholic acid (CDCA), the primary bile acids. In this pathway, microsomal sterol 12 $\alpha$ -hydroxylase (CYP8B1) is required for the synthesis of cholic acid and mitochondrial sterol 27-hydroxylase for side-chain oxidation. The alternative pathway is initiated by mitochondrial sterol 27-hydroxylase (CYP27A1), followed by oxysterol 7 $\alpha$ -hydroxylase (CYP7B1) in extrahepatic tissues. Oxysterol metabolites are then transported from peripheral tissues into hepatocytes and further conversion to CDCA. These four cytochrome P450 genes have recently been cloned and molecular mechanisms of regulation of these major regulatory genes in bile acid biosynthesis are currently under study. Several inborn errors of bile acid biosynthesis have been identified. Advances in molecular genetics of bile acid biosynthesis have led to a better understanding of the molecular basis of regulation of bile acid synthesis, the mechanisms of pathogenesis of cholestatic liver diseases, and the therapy for treatment of hypercholesterolemia and cholestatic liver diseases.

**KEY WORDS:** cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), oxysterol 7 $\alpha$ -hydroxylase (CYP7B1), sterol 27-hydroxylase (CYP27A1), sterol 12 $\alpha$ -hydroxylase (CYP8B1), cholestasis, atherosclerosis.

### INTRODUCTION

The liver plays an important role in cholesterol homeostasis. A free cholesterol pool in hepatocytes is maintained by several input and output mechanisms (Figure 4.1). Excess cholesterol in extrahepatic tissues is excreted into the circulation and taken up into hepatocytes as chylomicron remnants by low density lipoprotein (LDL) receptors or LDL-related



**Figure 4.1 Cholesterol metabolism in the liver.** Cholesterol homeostasis in the liver is maintained by several input and output mechanisms. Cholesterol input is maintained by uptake of cholesterol and cholesterol esters by LDL, HDL SRB1 and LRP receptors, and *de novo* synthesis from acetyl-CoA that is regulated by rate-limiting enzyme HMG-CoA reductase. The output mechanism is maintained by conversion of cholesterol to bile acids, and esterification of cholesterol by acyl-CoA: cholesterol acyltransferase (ACAT). Bile acids are secreted into bile, stored in gallbladder and excreted into intestine. Bile acids carry cholesterol to intestine and excrete cholesterol to feces. Most bile acids are recirculated to hepatocyte via portal circulation and inhibit the rate-limiting enzyme of bile acid synthesis, cholesterol 7 $\alpha$ -hydroxylase (CYP7A1).

protein (LRP), or by reverse cholesterol transport of high density lipoprotein (HDL) by the scavenger receptor SR-BI (Kozarsky *et al.*, 1997; Rigotti *et al.*, 1997). Liver also synthesizes cholesterol from acetyl-CoA 3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase is the rate-limiting enzyme in this pathway. Output of cholesterol is mainly by conversion of cholesterol to bile acids, which accounts for the catabolism of about 50% of cholesterol in the body. Bile acids (or bile salts, the physiological form) form mixed micelles with cholesterol and phospholipids, which are excreted into bile and stored in gallbladder. After each meal, bile acids are secreted into the intestine. In the intestine, bile acids facilitate the disposal of about 40% of cholesterol into feces, and absorption of fats and lipid-soluble vitamins. In this process, bile acids are quantitatively reabsorbed in the ileum by active bile acid transport systems (Dawson and Oelkers, 1995). Bile acids transported via the portal

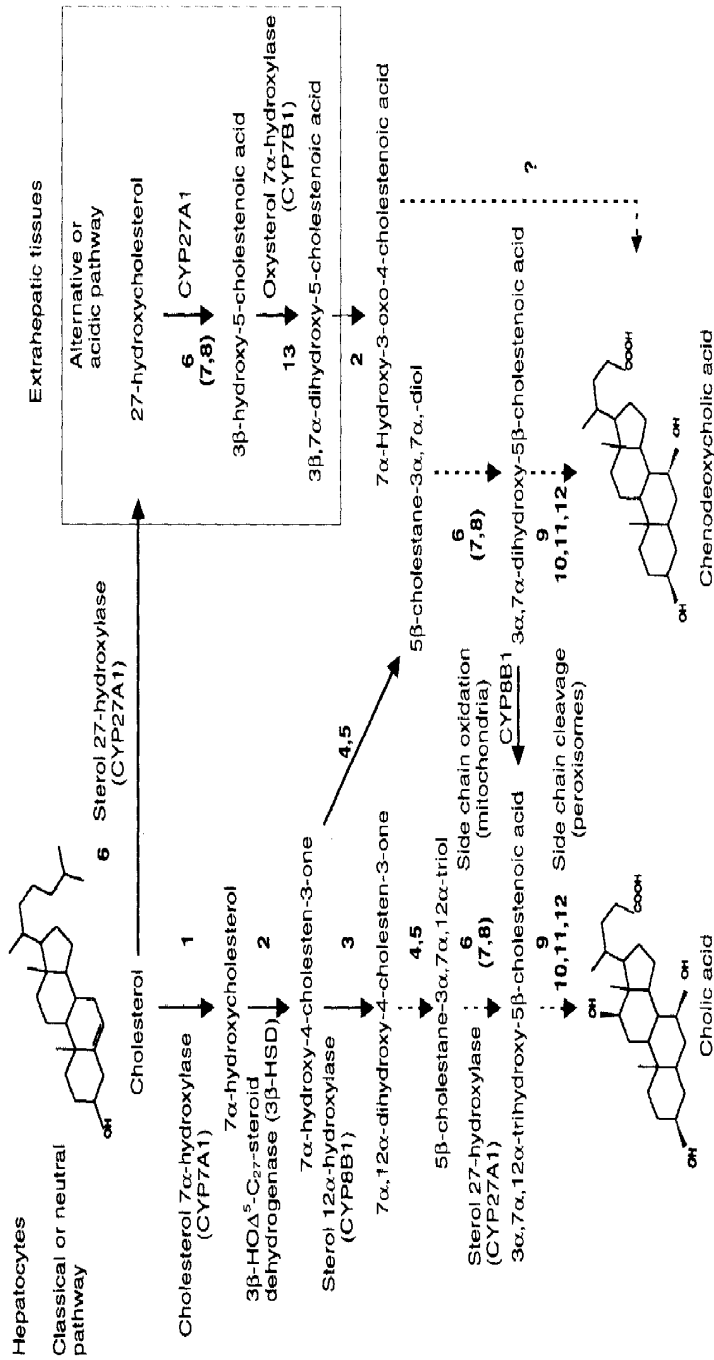
venous circulation are taken up by the  $\text{Na}^+$ -taurocholate co-transporter and  $\text{Na}^+$ -independent organic anion transporting polypeptide, which are located in the sinusoidal membrane (basolateral) of the liver. In hepatocytes, bile acids bind to bile acid binding proteins, are shuttled vectorially across the hepatocytes to the canalicular surface, and secreted into the bile canalicular (apical) by the canalicular multispecific organic anion transporter and ATP-dependent bile salt transporter (Suchy *et al.*, 1997; Trauner *et al.*, 1998). This enterohepatic circulation of bile plays important physiological roles in absorption and distribution of nutrients and is remarkably efficient, with only 5% bile acids lost in feces which is compensated by biosynthesis from cholesterol. Bile acid biosynthesis pathways thus play important roles in maintaining cholesterol homeostasis in mammals. The remaining 10% cholesterol is converted to cholesterol esters by acyl-CoA: cholesterol acyltransferase (ACAT), which are excreted as VLDL and HDL to extrahepatic tissues for the synthesis of steroid hormones and membranes.

### BILE ACID BIOSYNTHESIS PATHWAYS

Cholesterol degradation to primary bile acids can be initiated by either cholesterol 7 $\alpha$ -hydroxylase of the classical (or neutral) pathway, or by mitochondrial sterol 27-hydroxylase of the alternative (or acidic) pathway (Bjorkhem, 1985; Chiang, 1998; Chiang and Vlahcevic, 1996; Javitt, 1994; Princen *et al.*, 1997; Russell and Setchell, 1992) (Figure 4.2). In the neutral pathway, modifications of the cholesterol nucleus including saturation of the double bond, epimerization of the 3 $\beta$ -hydroxyl group, and hydroxylations at 7 $\alpha$  and 12 $\alpha$ -position precede oxidative cleavage of the side chain. The end products of cholesterol degradation via the neutral pathway are cholic and chenodeoxycholic acids, which are synthesized in a ratio of about 2 to 1 in humans. The acidic pathway predominantly produces chenodeoxycholic acid (Javitt *et al.*, 1989). The relative contribution of these two pathways of bile acid biosynthesis to the total bile acid synthesis under physiological conditions in man is not clear and is a subject of much debate (Javitt, 1994). It is likely that the classical pathway is the main pathway, which is highly regulated under physiological conditions, and becomes the predominant pathway when bile acid feedback is interrupted, i.e., treatment with bile acid sequestrant or during chronic biliary diversion. Whereas the alternative pathway contributes very little to overall bile acid synthesis under normal conditions, it becomes the major pathway to compensate for the low rate of bile acid synthesis in certain liver diseases (Axelson and Sjoval, 1990; Del Puppo *et al.*, 1998). In cultured human and rat hepatocytes, the alternative pathway may account for nearly 50% of total bile acid synthesis (Princen *et al.*, 1991). When cholesterol synthesis was blocked with squalenstatin to inhibit cholesterol 7 $\alpha$ -hydroxylase in bile fistula rats, bile acid synthesis was maintained, presumably by stimulation of the alternative pathway (Vlahcevic *et al.*, 1997). Other minor bile acid biosynthesis pathways, such as microsomal 25-hydroxylase pathway may also be involved in cholic acid synthesis (Bjorkhem, 1985). Thus, the presence of

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**Figure 4.2 Bile acid biosynthesis pathways.** Two major pathways of bile acid biosynthesis are shown. The classical or neutral pathway is located in hepatocytes. The alternative or acid pathway is also located in extrahepatic tissues. Only major enzymes and intermediates are shown. See Table 4.1 for details enzyme names, reaction catalyzed, and subcellular location of each step in bile acid biosynthesis pathways. Bold numbers refer to enzymatic reactions, which are listed in Table 4.1.



multiple bile acid biosynthesis pathways underscores the importance of bile acid biosynthesis in liver metabolism and cholesterol homeostasis. Two major bile acid biosynthesis pathways, the classical or neutral pathway and the alternative or acidic pathway are shown in Figure 4.2. Table 4.1 summarizes enzymes and reactions in two major bile acid biosynthesis pathways.

The classical (or neutral) pathway

The biosynthesis of bile acids from cholesterol via the neutral pathway requires thirteen different reactions catalyzed by twelve different enzymes located in the cytoplasm, microsomes, mitochondria and peroxisomes (Table 4.1). This pathway is initiated by cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) which converts cholesterol to 7 $\alpha$ -hydroxycholesterol. This microsomal enzyme is the only rate-determining enzyme in the bile acid biosynthetic pathway (Myant and Mitropoulos, 1977). The 7 $\alpha$ -hydroxycholesterol formed is converted to 7 $\alpha$ -hydroxy-4-cholesten-3-one by a microsomal 3 $\beta$ -hydroxy- $\Delta^5$ -C<sup>27</sup>-steroid dehydrogenase/ isomerase (3 $\beta$ -HSD) (Bjorkhem, 1985). 7 $\alpha$ -hydroxy-4-cholesten-3-one is a branch point intermediate in the bile acid biosynthesis pathway. For the synthesis of chenodeoxycholic acid, 7 $\alpha$ -hydroxy-4-cholesten-3-one is reduced to 5 $\beta$ -cholestane-3 $\alpha$ , 7 $\alpha$ -diol by two cytosolic enzymes  $\Delta^{4-3}$ -oxosteroid-5 $\beta$ -reductase and 3 $\alpha$ -hydroxysteroid dehydrogenase (Figure 4.2). For the synthesis of cholic acid, 7 $\alpha$ -hydroxy-4-cholesten-3-one is first hydroxylated at the C-12 position by a microsomal sterol 12 $\alpha$ -hydroxylase (CYP8B1) and then reduced to 5 $\beta$ -cholestane-3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -triol. This sequence of reactions completes the modification of the sterol nucleus of cholesterol.

The initial step in the side-chain oxidation is 27-hydroxylation of the methyl group by mitochondrial sterol 27-hydroxylase (CYP27A1) (Figure 4.2). Sterol

Table 4.1 Enzymes catalyzing conversion of cholesterol to bile acids.

Enzyme	Reaction catalyzed	Subcellular localization
1. Cholesterol 7 $\alpha$ -hydroxylase	C7-hydroxylation	ER
2. 3 $\beta$ -hydroxy- $\Delta^5$ -C <sup>27</sup> steroid dehydrogenase/isomerase	Oxidation 3 $\beta$ -OH to 3-oxo and $\Delta^5$ to $\Delta^4$	ER
3. Sterol 12 $\alpha$ -hydroxylase	C12-hydroxylation	ER
4. $\Delta^{4-3}$ -oxosteroid 5 $\beta$ -reductase	Saturation double bond	Cytoplasm
5. 3 $\alpha$ -hydroxysteroid dehydrogenase	Reduction 3-oxo to 3 $\alpha$ -OH	Cytoplasm
6. Sterol 27-hydroxylase	Side-chain oxidation CH <sub>3</sub> to CH <sub>2</sub> OH to COOH	Mitochondria
7. Alcohol dehydrogenase	Side-chain oxidation CH <sub>2</sub> OH to CHO	Cytoplasm
8. Aldehyde dehydrogenase	Side-chain oxidation CHO to COOH	Cytoplasm
9. Bile acid coenzyme A ligase	Side-chain oxidation Ligation of CoA	ER
10. Acyl-CoA oxidase	Side-chain oxidation to enoyl CoA	Peroxisomes
11. Acyl-CoA hydratase/dehydrogenase	Side-chain oxidation to keto-acyl CoA	Peroxisomes
12. Acyl-CoA thiolase peroxisomes	Side-chain cleavage	
13. Oxysterol 7 $\alpha$ -hydroxylase	C7-hydroxylation	ER

Note  
Modified from Balistreri (1995).

27-hydroxylase can both hydroxylate and carboxylate the C<sub>27</sub> methyl group (Russell and Setchell, 1992). Alternatively, cytosolic alcohol and aldehyde dehydrogenases may also catalyze these two reactions. The product 3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5 $\beta$ -cholestanoic acid (THCA) or 3 $\alpha$ ,7 $\alpha$ -dihydroxy-5 $\beta$ -cholestanoic acid (DHCA) is ligated to coenzyme A by an ATP-dependent microsomal bile acid coenzyme A ligase (Bjorkhem, 1985). The two cholestanoyl-CoAs are subsequently transported into peroxisomes and their side chains are shortened by one cycle of  $\beta$ -oxidation. In  $\beta$ -oxidation, peroxisomal acyl-CoA oxidase converts acyl-CoA to enoyl-CoA that is then converted to keto acyl-CoA by peroxisomal bifunctional enzyme acyl-CoA hydratase/dehydrogenase. Peroxisomal acyl-CoA thiolase then cleaves C3 unit as propionyl-CoA and generates chenodeoxycholoyl-CoA and choloyl-CoA. These two compounds are conjugated at C<sub>24</sub> to either glycine or taurine by bile acid-CoA: amino acid N-acyltransferase prior to secretion into bile. Primary bile acids, CA and CDCA, are converted to secondary bile acids, deoxycholic acid and lithocholic acid, respectively, by bacterial 7 $\alpha$ -dehydroxylase in the intestine.

### The alternative (or acidic) pathway

Bile acid biosynthesis via the acidic pathway is initiated by sterol 27-hydroxylase (CYP27A1) to convert cholesterol to 27-hydroxycholesterol and 3 $\beta$ -hydroxy-5-cholestenoic acid (Figure 4.2). Both 27-hydroxycholesterol and 3 $\beta$ -hydroxy-5-cholestenoic acid are 7 $\alpha$ -hydroxylated by microsomal oxysterol 7 $\alpha$ -hydroxylase (CYP7B1) to 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholestenoic acid (Björkhem *et al.*, 1992; Shoda *et al.*, 1993). This oxysterol 7 $\alpha$ -hydroxylase is distinct from cholesterol 7 $\alpha$ -hydroxylase (Martin *et al.*, 1993). It also converts 27-hydroxycholesterol to 7 $\alpha$ ,27-dihydroxy cholesterol (Martin *et al.*, 1997; Schwarz *et al.*, 1997). Microsomal 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub> steroid dehydrogenase/isomerase then converts 3 $\alpha$ ,7 $\alpha$ -dihydroxy-5-cholestenoic acid to 7 $\alpha$ -hydroxy-3-oxo-4-cholestenoic acid, a precursor of chenodeoxycholic acid. Little is known about remaining enzymes involved in the conversion of this metabolite to chenodeoxycholic acid. Recent observation that both cholic acid and chenodeoxycholic acid can be formed when cholesterol 7 $\alpha$ -hydroxylase is inhibited in rat hepatocytes provided suggestive evidence that a pathway might also hydroxylate intermediates at the 12 $\alpha$ -position to produce cholic acid via the acidic pathway (Vlahcevic *et al.*, 1997). Rabbit sterol 12 $\alpha$ -hydroxylase is able to 12 $\alpha$ -hydroxylate 3 $\alpha$ ,7 $\alpha$ -dihydroxy-5-cholestanoic acid, an intermediate in CDCA synthesis, and lead to the synthesis of cholic acid (Andersson *et al.*, 1998) (Figure 4.2).

### Regulation of bile acid biosynthesis

Enterohepatic recirculation of bile acids is the most important physiological regulation of the rate of bile acid biosynthesis (Chiang, 1998). This feedback mechanism not only regulates bile acid biosynthesis by inhibiting the rate-limiting enzyme, cholesterol 7 $\alpha$ -hydroxylase, but also down-regulates cholesterol synthesis by inhibiting the rate-limiting enzyme, HMG-CoA reductase. Interruption of bile acid feedback by bile acid sequestrants, i.e.,

cholestyramine, results in stimulation of both cholesterol synthesis by up-regulating HMG-CoA reductase and the rate of bile acid synthesis by de-repression of cholesterol 7 $\alpha$ -hydroxylase.

The rate of overall bile acid biosynthesis is regulated by bile acids at the rate-limiting enzyme of the neutral pathway, cholesterol 7 $\alpha$ -hydroxylase. Bile acid feedback also regulates other regulatory enzymes in the pathway, oxysterol 7 $\alpha$ -hydroxylase, sterol 12 $\alpha$ -hydroxylase, and 3 $\beta$ -HSD. It has been well established that the rate of bile acid synthesis and cholesterol 7 $\alpha$ -hydroxylase activity is regulated by the hydrophobicity index of bile. Hydrophobic bile acids are more potent inhibitors of bile acid biosynthesis than hydrophilic bile acids (Heuman *et al.*, 1989). Sterol 12 $\alpha$ -hydroxylase may regulate hydrophobicity of bile by regulating the ratio of cholic acid to chenodeoxycholic acid.

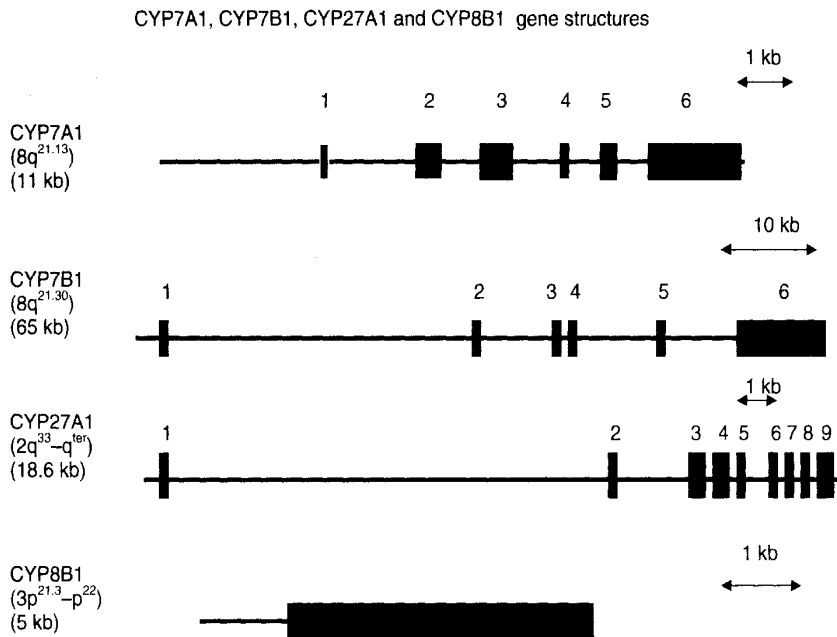
The mechanism by which the acidic pathway is regulated is not clear. Both sterol 27-hydroxylase and oxysterol 7 $\alpha$ -hydroxylase are potentially regulated. Some evidence suggests that sterol 27-hydroxylase is regulated by bile acid feedback (Vlahcevic *et al.*, 1996). Other reports suggest that 27-hydroxylase is not regulated by bile acids (Araya *et al.*, 1995), but by cholesterol (Xu *et al.*, 1999).

### Oxysterols

Oxysterols found *in vivo* are oxidized C27 sterols (Brown and Jessup, 1999). 7 $\alpha$ -hydroxycholesterol, 7 $\beta$ -hydroxycholesterol, 7-ketocholesterol, 27-hydroxycholesterol and 25-hydroxycholesterol are the most common oxysterols found in human serum, macrophages and vascular endothelium, and are especially enriched in atherosclerotic plaques. When cholesterol levels become high, oxysterols are generated either by auto-oxidation of cholesterol or hydroxylation of sterol hydroxylases. Most oxysterols are produced *in vivo* by enzymes in cholesterol catabolism, such as sterol 27-hydroxylase, sterol 25-hydroxylase and cholesterol 7 $\alpha$ -hydroxylase. Oxysterols are potent inhibitors of LDL receptor as well as HMG-CoA reductase and other enzymes in the mevalonate pathway. Thus, sterol hydroxylases in bile acid biosynthesis pathways play important roles in cholesterol homeostasis. Because oxysterol 7 $\alpha$ -hydroxylase has broad substrate specificity for various oxysterols, it may metabolize cytotoxic oxysterols and play important roles in regulating oxysterol levels. Oxysterols formed in extrahepatic tissues are diffused into the circulation, transported into the liver, and are converted to bile acids presumably by the acidic pathway. Oxysterols may function in a signaling pathway, which regulates cholesterol and bile acid biosynthesis, and play roles in maintaining cholesterol homeostasis.

### MAJOR REGULATORY ENZYMES IN BILE ACID BIOSYNTHESIS

Four cytochrome P450 enzymes play important roles in biosynthesis of bile acids and oxysterols. Cholesterol 7 $\alpha$ -hydroxylase perhaps is the most important regulatory enzyme in bile acid biosynthesis pathways and has been studied extensively in the last thirty years. A detailed update of the structure and regulation of the cholesterol 7 $\alpha$ -hydroxylase gene is presented. Sterol 27-hydroxylase mutations have been identified in the cholesterol storage disease, cerebrotendinous xanthomatosis (CTX). Sterol 12 $\alpha$ -hydroxylase may play a role in



**Figure 4.3 Structures of P450 genes in bile acid biosynthesis.** Four cytochrome P450 genes, CYP7A1, CYP7B1, CYP27A1 and CYP8B1 are involved in bile acid biosynthesis pathways. Filled boxes represent exons, which are numbered above each exon. Thin lines between two exons are introns. The sizes of exons in CYP7B1 and CYP27A1 are not drawn to scale. Chromosomal location and the size of each gene are shown.

regulation of cholic acid synthesis. The CYP8B1 cDNA and gene were cloned only recently. The presence of a second  $7\alpha$ -hydroxylase in bile acid biosynthesis pathways has been suggested for many years. More recently, the cDNA most closely related to cholesterol  $7\alpha$ -hydroxylase was cloned from a hippocampus cDNA library and was later shown to be able to  $7\alpha$ -hydroxylate various oxysterols. This enzyme was named as oxysterol  $7\alpha$ -hydroxylase.

Figure 4.3 shows the structures of the CYP7A1, CYP7B1, CYP27A1 and CYP8B1 genes. The CYP7A1 and CYP7B1 genes are closely localized on chromosome 8 and share the same exon-intron structures. The CYP7A1 gene spans about 11kb of the genome whereas CYP7B1 is much larger, about 65kb. The CYP27A1 gene has nine exons and spans about 18.6kb on chromosome 2. Interestingly, CYP8B1 is intron-less and is located on chromosome 3.

### Cholesterol $7\alpha$ -hydroxylase

Cholesterol  $7\alpha$ -hydroxylase is a unique cytochrome P450 isozyme that has strict substrate specificity for cholesterol and is only expressed in the liver. Studies on the regulation of bile acid synthesis have exclusively focused on the regulation of cholesterol  $7\alpha$ -hydroxylase activity in microsomes of normal and bile fistula rat models. Recently the CYP7A1 cDNAs

and genes were cloned and the study of the molecular basis of regulation of this rate-limiting enzyme became possible (Noshiro *et al.*, 1989; Jelinek *et al.*, 1990; Li *et al.*, 1990; Karam and Chiang, 1992; Crestani *et al.*, 1993). The mRNA (4 kb) translates a polypeptide of 503 (rat) or 504 (human) amino acid residues. The CYP7A1 gene consists of six exons and five introns (Figure 4.3). The gene was previously mapped to q11-q12 of the human chromosome 8 (Cohen *et al.*, 1992) and mouse chromosome 4 (Machleder *et al.*, 1997). The location of the CYP7A1 gene was recently mapped to 8q21.13 and closely linked to the D8S1113 marker (Setchell *et al.*, 1998). Sequences from the transcription start site to about 250 bp upstream are highly homologous among different species (Jelinek and Russell, 1990; Chiang *et al.*, 1992; Cohen *et al.*, 1992; Molowa *et al.*, 1992; Crestani *et al.*, 1993; Poorman *et al.*, 1993; Thompson *et al.*, 1993; Tzung *et al.*, 1994; Wang and Chiang, 1994). The CYP7A1 promoter contains a TATA box sequence located about 30bp upstream of the transcription start site (+1).

Mice deficient of CYP7A1 gene have been obtained by gene knockout (Ishibashi *et al.*, 1996). These mice displayed complex phenotypes including oily coats, hyperkeratosis, vision defects, and behavioral irregularities, consistent with malabsorption of vitamins E and D<sub>3</sub>. Homozygous animals (CYP7A1<sup>-/-</sup>) died within 18 days; 40% of them died between days 1 and 4, and 45% died within days 11 and 18. Vitamin supplement to nursing mothers could prevent deaths in the early period and bile acid supplement prevented deaths in the later period. CYP7A1<sup>-/-</sup> mice have low levels of vitamin E and D<sub>3</sub> and elevated stool fat content (Schwarz *et al.*, 1996). However, several 7 $\alpha$ -hydroxylated bile acids were detected in the bile and stool of adult CYP7A1<sup>-/-</sup> mice. This was explained by the expression of a hepatic oxysterol 7 $\alpha$ -hydroxylase activity in the liver after weaning (21 days) that accounted for the synthesis of abnormal 7 $\alpha$ -hydroxylated bile acids in these mice. The new born (5 to 10 days old) CYP7A1<sup>-/-</sup> mice developed neonatal cholestasis due to accumulation of monohydroxylated bile acids, 3 $\beta$ -hydroxy-5-cholenoate and 3 $\alpha$ -hydroxy-5 $\beta$ -cholanoate, and 27-hydroxycholesterol, due to the lack of oxysterol 7 $\alpha$ -hydroxylase expressed three weeks after birth (Arnon *et al.*, 1998).

#### *Regulation of cholesterol 7 $\alpha$ -hydroxylase*

Cholesterol 7 $\alpha$ -hydroxylase activity is mainly regulated by its substrate, cholesterol, and the end products, bile acids. Hydrophobic bile acids such as deoxycholic acid (DCA) and chenodeoxycholic acid are potent repressors of cholesterol 7 $\alpha$ -hydroxylase activity, whereas hydrophilic bile acids such as ursodeoxycholic acid (UDCA) do not have much effect (Bertolotti *et al.*, 1991; Heuman *et al.*, 1989; Shefer *et al.*, 1990; Xu *et al.*, 1992). It is well established that bile acids are the most important physiological regulators, and they regulate cholesterol 7 $\alpha$ -hydroxylase predominantly at the gene transcriptional level (Chiang, 1998).

Dietary cholesterol regulates cholesterol 7 $\alpha$ -hydroxylase differently in different species. A high cholesterol diet stimulates cholesterol 7 $\alpha$ -hydroxylase expression in the rat and mouse. In contrast, a high cholesterol diet suppressed cholesterol 7 $\alpha$ -hydroxylase activity and mRNA levels in African green monkeys (Rudel *et al.*, 1994) but had no effect on hamsters (Horton *et al.*, 1995). In Watanabe rabbits, a model of familial hypercholesterolemia with defective LDL receptors, cholesterol 7 $\alpha$ -hydroxylase activity was found to be much lower

than that in New Zealand white rabbits and a high cholesterol diet did not further reduce enzyme activity (Xu *et al.*, 1995). In hypercholesterolemia-resistant rabbits (CRT/mlo), cholesterol 7 $\alpha$ -hydroxylase activity and mRNA levels were substantially higher than in normal New Zealand rabbits (Poorman *et al.*, 1993). It seems that the response to dietary cholesterol input varies greatly with different species and individuals (Kern, 1991). Rats and mice have the unique capability of maintaining cholesterol balance and are relatively resistant to diet-induced hypercholesterolemia.

The newly synthesized cholesterol may be the preferred substrate for cholesterol 7 $\alpha$ -hydroxylase (Myant and Mitropoulos, 1977; Straka *et al.*, 1990). Cholesterol synthesis has been found to link to regulation of cholesterol 7 $\alpha$ -hydroxylase, because inhibition of HMG-CoA reductase by lovastatin leads to the downregulation of CYP7A1 gene transcription, and mevalonate, a precursor of cholesterol, could prevent down-regulation in bile fistula rats (Jones *et al.*, 1993). In addition, squalostatins or zaragozic acid A, an inhibitor of squalene synthase, and AY9994, an inhibitor of 7-dehydrocholesterol  $\Delta^7$ -reductase in the cholesterol synthesis pathway, strongly inhibited cholesterol 7 $\alpha$ -hydroxylase activity and mRNA levels in rat liver or primary rat hepatocytes (Doerner *et al.*, 1995; Ness *et al.*, 1994; Pandak *et al.*, 1990).

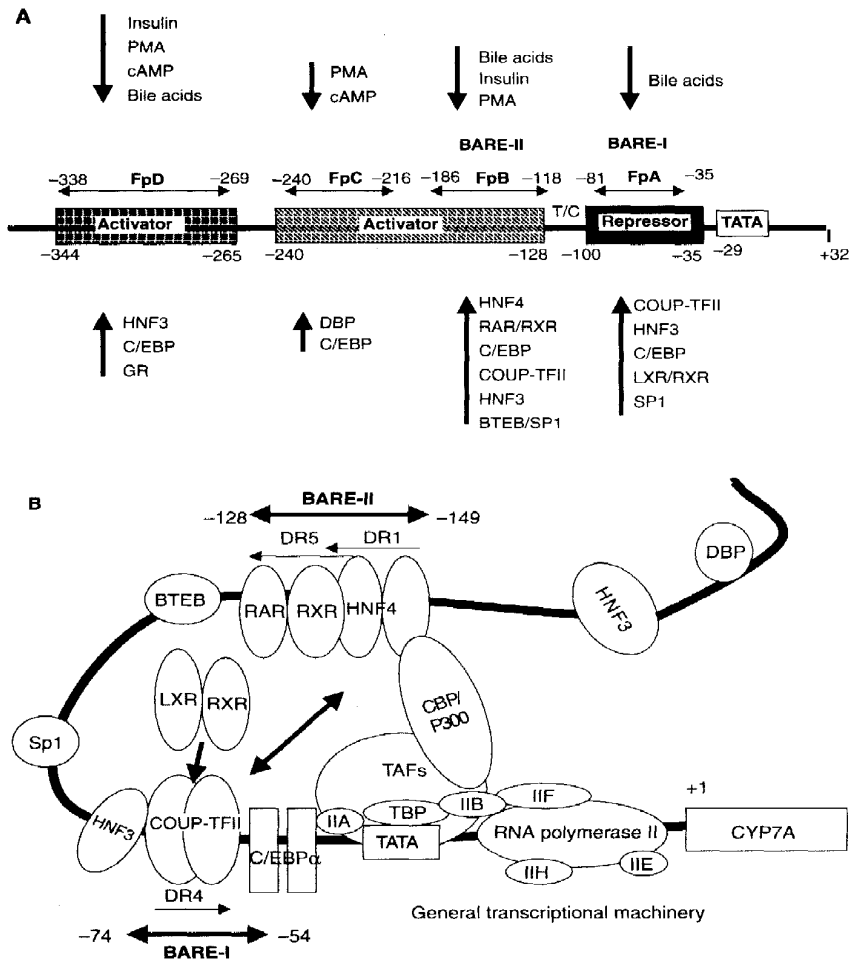
Taurocholate repressed cholesterol 7 $\alpha$ -hydroxylase gene transcription even in the presence of excess mevalonate (Pandak *et al.*, 1992; Stravitz *et al.*, 1993). In inbred strains of mice, taurocholate suppressed both the activity and mRNA of cholesterol 7 $\alpha$ -hydroxylase with or without high cholesterol diets (Dueland *et al.*, 1993). Therefore, the repressive effect of taurocholate overcomes the stimulative effect of cholesterol on 7 $\alpha$ -hydroxylase activity and mRNA. It is possible that dietary or *de novo* synthesized cholesterol may be required for a basal level of CYP7A1 gene expression while the hydrophobic bile acids play a regulatory role in modulating the level of CYP7A1 gene expression *in vivo*.

Several hormones including thyroid hormone (Ness *et al.*, 1990, 1994) and dexamethasone (Pandak *et al.*, 1997; Princen *et al.*, 1989) stimulated cholesterol 7 $\alpha$ -hydroxylase activity and mRNA levels, and transcriptional activity. In contrast, glucagon and cAMP repressed cholesterol 7 $\alpha$ -hydroxylase in rat primary hepatocytes (Hylemon *et al.*, 1992; Stravitz *et al.*, 1993). Both positive and negative cAMP regulatory regions were mapped in the rat promoter (Crestani *et al.*, 1995). Growth hormones stimulated cholesterol 7 $\alpha$ -hydroxylase activity without affecting mRNA levels in the rat (Rudling *et al.*, 1997). Expression of cholesterol 7 $\alpha$ -hydroxylase activity in the liver is the highest in the middle of the dark and lowest in the middle of the light cycle. The circadian rhythm is free running and is independent of external stimuli such as light and feeding. The variation of activity paralleled the levels of protein, mRNA and transcription of the CYP7A1 gene (Berkowitz *et al.*, 1995; Li *et al.*, 1990). Fibrates reduced cholesterol 7 $\alpha$ -hydroxylase activity in gallstone patients (Stahlberg *et al.*, 1994) and in hyperlipoproteinemia patients (Bertolotti *et al.*, 1995). Thus, the CYP7A1 gene is regulated by multiple hormones, and the mechanisms of regulation by these physiological stimuli are extremely complicated.

*Regulation of the CYP7A1 gene transcription*

Potential regulatory sequences in the 5'-flanking region of the CYP7A1 gene have been identified by DNase I footprinting assay, transient transfection assay of CYP7A1 promoter/reporter gene activities, and electrophoretic mobility shift assay (EMSA). DNase I footprinting experiments revealed several protected footprints in the 5'-flanking region of the rat CYP7A1 gene (Chiang and Stroup, 1994). Figure 4.4A illustrates the structure of the rat CYP7A1 gene promoter. A repressor region was mapped between nt -100 and -35, since deletion of this sequence greatly stimulated CYP7A gene promoter/reporter activity (Chiang and Stroup, 1994). A *Msp*I fragment (-240 to -118) was identified as an activator, since deletion of this sequence almost completely abolished promoter activity (Crestani *et al.*, 1998). Another activator region (-344 to -265) was identified, because the nt -344 to +24/reporter construct has the highest transcriptional activity. A footprint (FpA, nt -81 to -35) contains consensus binding sites for liver-enriched hepatocyte nuclear factors HNF3 and HNF1 and a reversed CAAT box (Chiang and Stroup, 1994). An imperfect direct repeat (DR) of hormone response elements (HRE, AGG/TTCA) separated by four bases (DR4) is located in FpA. This DR4 is not a thyroid hormone response element, but a binding site for chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), which strongly stimulated rat CYP7A1 gene promoter activity (Stroup *et al.*, 1997). COUP-TFII is an orphan nuclear receptor important for embryogenesis and development. COUP-TFII can either negatively regulate genes by antagonizing other transcription factors or positively regulate genes by interaction with other transcription factors (Cooney *et al.*, 1992; Hall *et al.*, 1995; Kimura *et al.*, 1993; Ktistaki and Talianidis, 1997). Recently, the DR4 motif of the rat CYP7A1 gene promoter has been suggested as a binding site for LXR (liver orphan receptor) which transactivates a reporter fusion gene construct containing four copies of the DR4 motif of the rat CYP7A1 gene in CV-1 cells (Lehmann *et al.*, 1997). LXR is a nuclear receptor activated by binding to oxysterols (Janowski *et al.*, 1996; Peet *et al.*, 1998; Willy *et al.*, 1995). LXR can act as either a positive or negative regulator by binding to metabolites in the mevalonate pathway (Forman *et al.*, 1997). It was suggested that LXR might mediate an oxysterol signaling pathway in the regulation of lipid metabolism (Lehmann *et al.*, 1997). In mice lacking LXR, CYP7A1 mRNA levels were expressed normally in the liver, but were not stimulated by a high cholesterol diet as in wild type mice (Peet *et al.*, 1998). The lack of stimulation of bile acid synthesis by high cholesterol diet in *Lxr*<sup>-/-</sup> mice was thought to cause massive accumulation of cholesterol in the liver. LXR might function as a cholesterol sensor, which stimulates CYP7A1 expression. A *Spl* binding site was mapped to the -100/-82 region that was suggested previously as a basal transcription element binding protein (BTEB) binding site based on sequence analysis (Cohen *et al.*, 1992). *Spl* is a ubiquitous activator, which interacts with the general transcription machinery and regulates RNA polymerase II activity.

Sequences located between -186 and -118 (FpB) are the most conserved region of the promoter. It contains three HREs which form overlapping direct repeats spaced by one base (DR1) and five bases (DR5) (Crestani *et al.*, 1998). The DR5 motif has been identified as the binding site for nuclear receptors RXR/RAR, which strongly stimulated rat promoter activity. The DR1 motif has been mapped as a HNF4 binding site (Crestani *et al.*, 1998). HNF4 is an orphan nuclear receptor, which binds to DNA as homodimer (Jiang *et al.*, 1995).



**Figure 4.4 Structure of the rat cholesterol 7 $\alpha$ -hydroxylase gene promoter.** (A) Regulatory elements in proximal promoter of the rat CYP7A1 gene. Repressor and activators are shown in boxes. Four DNase I footprints (Fp) are localized. Negative regulatory factors regulate the gene through regions indicated by downward arrows. Positive regulatory factors regulate the gene through regions indicated by upward arrows. Bile acid response elements, BARE-I and BARE-II, are located in FpA and FpB, respectively. See text for details. (B) A model of transcription factors bound to the rat CYP7A1 promoter. Two bile acid response elements (BARE) are located in the proximal promoter. Nuclear hormone receptors HNF-4 and COUP-TFII bind to BARE-II and BARE-I, respectively, and synergistically activate gene transcription. In BARE-II, HNF4 and RAR/RXR bind to overlapping DR1 and DR5, respectively, and compete for binding to BARE-II. LXR/RXR binds to and may compete with COUP-TFII for DR4 in BARE-I. The TC-rich region located between BARE-I and BARE-II may bend the DNA and loop BARE-II over BARE-I and facilitate the interactions. HNF-3, Sp1, BTEB, and C/EBP also transactivate the gene. Co-activator CBP/P300 may mediate interaction of nuclear receptors with general transcriptional machinery, which contains TATA box binding protein (TBP), TBP-associated factors (TAP), basal transcription factors TFIID, IIA, IIB, IIF, IIIH and IIE, and enhance RNA polymerase II activity.



and regulates the liver-specific expression of many genes in lipoprotein metabolism (Ginsburg *et al.*, 1995). Fatty acyl-CoA thioesters have been suggested to be the ligands of HNF4 (Hertz *et al.*, 1998). HNF4 and COUP-TFII synergistically stimulated the rat CYP7A1 gene promoter activity. HNF4 may be the most important transcription factor required for basal level expression of cholesterol 7 $\alpha$ -hydroxylase in the liver (Crestani *et al.*, 1998). A HNF3 binding site located in nt -175/-166 was required for both basal transcriptional activity and stimulation of the rat CYP7A1 gene promoter activity by retinoic acid (Crestani *et al.*, 1998). A BTEB binding site has been mapped to -122GGCCGGG-127 (Foti *et al.*, 1998). BTEB is a GC-box binding protein of the Sp 1 family of transcription factor which regulates many cytochrome P450 genes (Imataka *et al.*, 1992). Bile acids, insulin and PMA repressed CYP7A1 promoter activity and response elements were mapped in the FpB region (Figure 4.4A).

Bile acid synthesis and the expression of cholesterol 7 $\alpha$ -hydroxylase activity, mRNA and protein levels followed a strong diurnal pattern (Chiang *et al.*, 1990; Noshiro *et al.*, 1990). Several footprints were identified using recombinant albumin D-site binding protein (DBP), a PAR family of clock genes (Lavery and Schibler, 1993). The major DBP binding site was located in nt -240 and -216 (FpC) (Lavery and Schibler, 1993; Lee *et al.*, 1994). The expression of DBP followed a stringent circadian rhythm (Wuarin and Schibler, 1990). Another PAR gene human leukemia factor 43 (HLF43) also stimulated the CYP7A1 transcription (Falvey *et al.*, 1995). DBP is a member of the basic leucine zipper (bZIP) transcription factors enriched in the liver. DBP binding sites also bind C/EBP $\alpha$  and C/EBP $\beta$  (Lee *et al.*, 1994). It was suggested that DBP and C/EBP might compete for overlapping binding sites to determine the relative rate of basal versus diurnally regulated CYP7A1 gene expression (Lee *et al.*, 1994). FpC region also mediates PMA and insulin response. Sequences located between -338 and -269 (FpD) contain binding sites for positive regulators, HNF3 and C/EBP. Negative regulatory elements of insulin response sequence (IRS), PRS and cAMP response element (CRE) and bile acids were also localized (Crestani *et al.*, 1995, 1996) (Figure 4.4A).

Three DNase I footprints (-340/-317, -315/-306, and -276/-260) in the human CYP7A1 were mapped using rat liver nuclear extracts (Molowa *et al.*, 1992). These footprints contain HNF3 binding sites. Using HepG2 nuclear extracts, nine DNase I footprints (FPs 1 to 9) were mapped in the 5'-flanking regions of the human CYP7A1 gene (Cooper *et al.*, 1997). FP1, FP2, and PF3 exactly match the rat FpA; FP4 (-104/-91) contains a putative BTEB site. FP5 (-144/-125) and FP6 (-191/-174) match the rat FpB. FP7 (-227/-213) matches the rat FpC. FP8 (-286/-265) and FP9 (-341/-313) match the rat FpD. Despite the similarity of nucleotide sequences in the proximal promoter of the rat and human CYP7A1 genes, these two homologous genes are regulated differently as demonstrated by transient transfection assays of promoter/reporter chimeric genes in HepG2 cells (Wang *et al.*, 1996). The human gene is not stimulated by retinoic acid. Transcription factors such as HNF3, HNF4 and COUP-TFII strongly stimulated rat CYP7A1 gene promoter activity when co-transfected in HepG2 cells, but had no or much less effect on the human CYP7A1 gene promoter. On the other hand, the human CYP7A1 gene is repressed more by insulin, bile acids and PMA than the rat gene. These results may explain

the much lower levels of cholesterol 7 $\alpha$ -hydroxylase activity and mRNA expressed in the liver of human than rat.

DNA sequences important in mediating bile acid repression of CYP7A1 gene transcription have been identified. A bile acid response element-I (BARE-I) has been mapped to the sequence between nt -79 and -49 of the rat CYP7A1 gene (Chiang and Stroup, 1994; Hoekman *et al.*, 1993). Deletion of this sequence reduced, but not abolished, bile acid repression of CYP7A1 gene transcriptional activity. A second bile acid responsive element named BARE-II was recently mapped to a sequence between nt -149 and -118 (Stroup *et al.*, 1997). This BARE-II shares an identical AGTTCAAG core sequence with BARE-I. Mutations or deletion of nucleotides in BARE-II reduced bile acid response. BARE-II perhaps is the major bile acid response element. BARE-III also mediated repression by phorbol ester and insulin (Crestani *et al.*, 1996).

Figure 4.4B illustrates the transcription factors bound to the proximal promoter of the rat CYP7A1 gene. Interactions and combinatorial actions of these transcription factors with general transcriptional machinery may determine the rate of gene transcription catalyzed by RNA polymerase II.

#### *Molecular mechanisms of regulation*

Previously, Chiang and Stroup (1994) proposed that bile acids might bind to and activate a bile acid receptor (BAR) which enters into nuclei and interacts with a bile acid responsive protein (BARP). BARP may be a positive transcription factor required for basal level transcription of the CYP7A1. Interaction between BAR and BARP might prevent the binding of BARP to BARE, thus repressing CYP7A1 gene expression (Chiang, 1998). Recent results suggested that BAR and BARP might be liver-enriched transcription factors or orphan nuclear receptors (Stroup *et al.*, 1997a, b). Hydrophobic bile acids or their metabolites might act as ligands for orphan nuclear receptors, similar in this aspect to many biologically active lipids identified as endogenous ligands for peroxisome proliferator-activated receptor (Bocos *et al.*, 1995; Devchand *et al.*, 1996; Forman *et al.*, 1997; Keller *et al.*, 1993). Preliminary results suggested that orphan nuclear receptor FXR (farnesoid X receptor) might function as a bile acid receptor and mediate the down-regulation of CYP7A1 gene by binding to BARE or preventing the binding of other transcription factors, i.e., HNF4 and COUP-TFII, to their binding sites. In another model, hydrophobic bile acids may activate protein kinase Cs which initiate a signal transduction pathway leading to down-regulation of CYP7A1 gene transcription (Rao *et al.*, 1997; Stravitz *et al.*, 1995). However, the downstream signaling molecules mediating bile acid response have not been identified. These two models may be complementary to each other in that bile acid-mediated phosphorylation of transcription factors may lead to downregulation of CYP7A1 gene.

A receptor-mediated mechanism may be a better model to explain the observation that hydrophobicity of bile acids regulates CYP7A1 gene transcription. The hydroxyl groups in the steroid rings may form a hydrophilic microenvironment in the otherwise hydrophobic molecules (Princen *et al.*, 1997). The stereo-structure of a bile acid may determine its specificity for binding to a transcription factor (nuclear receptor) that interacts with regulatory sequences in the CYP7A1 gene.

### Sterol 27-hydroxylase

Sterol 27-hydroxylase has broad substrate specificity and is expressed in many tissues. In addition to 27-hydroxylation of cholesterol, sterol 27-hydroxylase is also able to metabolize 7 $\alpha$ -hydroxy-4-cholesten-3-one (Bjorkhem *et al.*, 1994), 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ ,12 $\alpha$ -triol (Pikuleva *et al.*, 1997), and C<sub>27</sub>-sterols (Bjorkhem *et al.*, 1994). Sterol 27-hydroxylase has been shown to catalyze 25-hydroxylation of vitamin D<sub>3</sub> (Usui *et al.*, 1990a, b). In pig liver, mitochondrial 27-hydroxylase is able to catalyze 27-, 25-, or 24-hydroxylation of cholesterol (Lund *et al.*, 1993). Human CYP27A1 is able to hydroxylate vitamin D analogs at 1 $\alpha$ -, 24-, 25-, or 27-position when expressed in COS cells (Guo *et al.*, 1993). Recently, a 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase cDNA has been cloned (Shinki *et al.*, 1997). The amino acid sequence shares 44% identity with vitamin D<sub>3</sub>-25-hydroxylase (CYP27A1) and 34% identity with 25-hydroxyvitamin D<sub>3</sub>-24-hydroxylase (CYP24). Thus, 25-hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -hydroxylase is a new subfamily (CYP27B1) of the CYP27 family and sterol 27-hydroxylase should be named as CYP27A1. Sterol 27-hydroxylase activity, mRNA and protein are present at high levels in vascular endothelium (Reiss *et al.*, 1994), atherosclerotic plaque (Crisby *et al.*, 1997; Reiss *et al.*, 1997), macrophages (Bjorkhem *et al.*, 1994) and fibroblasts (Zhang *et al.*, 1995). It has been proposed that the function of CYP27A1 might be to eliminate cholesterol in macrophages and endothelial cells (Babiker *et al.*, 1997). Oxidized cholesterol is released into serum for transport to the liver and conversion to bile acids. This may provide a defense against atherosclerosis in macrophages (Bjorkhem *et al.*, 1994) and in the arterial wall (Reiss *et al.*, 1997). The link of CYP27A1 mutations to CTX and oxysterols to the development of atherosclerosis and apoptosis suggests a major role for CYP27A1 in disease processes.

The rabbit, rat and human mitochondrial sterol 27-hydroxylases have been purified and cloned and the nucleotide sequences of the cDNAs determined (Andersson *et al.*, 1989; Usui *et al.*, 1990a; Cali *et al.*, 1991; Cali and Russell, 1991). The recombinant sterol 27-hydroxylase is able to further metabolize 27-hydroxycholesterol to 3 $\beta$ -hydroxy-5-cholestenoic acid (Pikuleva *et al.*, 1998). The sterol 27-hydroxylase gene has been mapped to q33-qter interval of human chromosome 2 (Cali and Russell, 1991). CYP27A1 contains nine exons and eight introns spanning at least 18.9kb of the genome (Figure 4.3) (Leitersdorf *et al.*, 1993). The enzyme is located in the inner mitochondrial membrane and requires ferredoxin, ferredoxin reductase and NADPH for activity. The 27-hydroxylase mRNAs (1.9kb) are present in high levels in the liver, duodenum and adrenal gland, and at much lower levels in the lung, kidney and spleen (Andersson *et al.*, 1989). The cDNA encodes a protein of 535 amino acid residues containing the N-terminal mitochondrial signal peptide of 36 amino acid residues. The promoter lacks a TATA-like sequence and is GC-rich.

It has been reported that transcription of the rat CYP27A1 is suppressed by bile acids, although to a much less extent than the CYP7A1 (Dahlback-Sjoberg *et al.*, 1993; Twisk *et al.*, 1995; Stravitz *et al.*, 1996; Vlahcevic *et al.*, 1996). However, rabbit CYP27A1 is neither repressed by bile acids nor stimulated by cholestyramine (Araya *et al.*, 1995). CYP27A1 activity was increased with increased bile acid synthesis in cholesterol-fed rabbits, but bile drainage did not affect CYP27A1 activity in cholesterol-fed rabbits (Xu *et al.*, 1996). Insulin also suppressed CYP27A1 transcription (Twisk *et al.*, 1995). Dexamethasone was required for the expression of sterol 27-hydroxylase activity in rat primary hepatocytes; however,

thyroxin did not affect sterol 27-hydroxylase expression (Stravitz *et al.*, 1996). It seems clear that the CYP27A1 gene is not regulated as much as the CYP7A1 gene. Xu *et al.* (1998, 1999) reported recently that a high cholesterol diet increased the bile acid pool size, which inhibited cholesterol 7 $\alpha$ -hydroxylase activity without an effect on sterol 27-hydroxylase activity in rabbits. In contrast, sterol 27-hydroxylase was stimulated by dietary cholesterol. These investigators pointed out that increasing dietary cholesterol induced the alternative bile acid synthesis pathway initiated by sterol 27-hydroxylase. Sterol 27-hydroxylase is insensitive to bile acid feedback in rabbits. Thus, these two rate-limiting enzymes in the classical and alternative bile acid synthesis pathways are regulated differently by bile acids and cholesterol. Regulatory elements on the CYP27A1 promoter have not been identified yet.

### Oxysterol 7 $\alpha$ -hydroxylase

The existence of a sterol 7 $\alpha$ -hydroxylase of 27- and 25-hydroxycholesterol in liver has been suggested (Bjorkhem *et al.*, 1992; Javitt, 1994; Martin *et al.*, 1993). Two liver microsomal cytochrome P450 fractions and a mitochondrial 7 $\alpha$ -hydroxylase fraction capable of 7 $\alpha$ -hydroxylation of 27-hydroxycholesterol, 3 $\beta$ -hydroxy-5-cholestenoic acid and 3 $\beta$ -hydroxy-5-cholenoic acid were isolated from pig liver (Axelson *et al.*, 1992; Toll *et al.*, 1992). 7 $\alpha$ -hydroxylase of 27-hydroxycholesterol has been found in microsomes and mitochondria of human liver (Bjorkhem *et al.*, 1992; Shoda *et al.*, 1993) and fibroblasts (Zhang *et al.*, 1995b), in rat ovary and brain (Zhang *et al.*, 1995a; Payne *et al.*, 1995) and in hamster liver (Martin *et al.*, 1993). Partially purified oxysterol 7 $\alpha$ -hydroxylase from pig liver microsomes could hydroxylate both 25- and 27-hydroxycholesterol (Toll *et al.*, 1994). Multiple 7 $\alpha$ -hydroxylase activities toward 27-hydroxycholesterol and DHEA in pig liver microsomes have been reported (Norlin and Wikvall, 1998).

A cDNA with sequence identity of 35% to CYP7A1 was isolated from a mouse hippocampus cDNA library (Stapleton *et al.*, 1995). This was the second gene identified in the CYP7 family and was designated as CYP7B1. Later, Martin *et al.* (1997) transfected kidney 293/T cells with mouse brain CYP7B1 expression plasmid and showed that 27-hydroxycholesterol was metabolized to cholest-5-ene-3 $\beta$ ,7 $\alpha$ ,27-triol. They also demonstrated that cholesterol synthesis was not inhibited by 27-hydroxycholesterol in cells overexpressing CYP7B1. The HeLa cell transfected with mouse brain CYP7B1 has been shown to catalyze 7 $\alpha$ -hydroxylation of neurosteroids, dehydroepiandrosterone (DHEA) and pregnenolone (Rose *et al.*, 1997). Significant DHEA 7 $\alpha$ -hydroxylase activity was observed in the primary rat brain and liver extracts. The brain CYP7B1 overexpressed in kidney 293 cells was shown to metabolize 25-hydroxycholesterol to cholest-5-ene-3 $\beta$ , 7 $\alpha$ ,25-triol (Schwarz *et al.*, 1997). These experiments established the identity of CYP7B1 as an oxysterol 7 $\alpha$ -hydroxylase with very broad substrate specificity.

Oxysterol 7 $\alpha$ -hydroxylase mRNAs were expressed at the highest level in the brain of the rat and mouse, and at much lower levels in the liver of male rats (Stapleton *et al.*, 1995). In contrast, Schwarz and colleagues (Schwarz *et al.*, 1998) reported that oxysterol 7 $\alpha$ -hydroxylase mRNAs were only expressed in liver and kidney, but not in the brain of mice. Dietary cholesterol and colestipol (a bile acid sequestrant) induced oxysterol 7 $\alpha$ -hydroxylase

activity, mRNA and protein levels in adult mouse liver (Schwarz *et al.*, 1998). High levels of oxysterol 7 $\alpha$ -hydroxylase activity were detected in mouse, hamster, rabbit and pig livers. An antibody against a 15 amino acid peptide of brain CYP7B1 cross-reacted with a polypeptide in mouse liver microsomes. The levels of this immuno-reacted peptide were induced after 18 days of life and correlated with oxysterol 7 $\alpha$ -hydroxylase activity and mRNA levels. In CYP7A<sup>(-/-)</sup> mice, bile acid pool size was reduced by 80%; however, no compensatory increase of oxysterol 7 $\alpha$ -hydroxylase was observed (Schwarz *et al.*, 1998). The activity is lower in the liver of female than male mice (Turley *et al.*, 1998). The specific activity of oxysterol 7 $\alpha$ -hydroxylase toward 25-hydroxycholesterol (6.7pmol/min/mg protein) was comparable to cholesterol 7 $\alpha$ -hydroxylase activity in male mouse liver (Turley *et al.*, 1998). This may suggest that oxysterol 7 $\alpha$ -hydroxylase is the rate-limiting enzyme subject to regulation in the acidic pathway of bile acid synthesis.

The size of human CYP7B1 mRNA was found to be about 9kb, which is much larger than that of rat and mouse (Wu and Chiang, unpublished results). Multiple mRNA northern blot analysis revealed that CYP7B1 was expressed at the highest levels in the brain, kidney, heart and pancreas, and at much lower levels in the liver, lung, skeletal muscle and placenta. This pattern of mRNA expression is similar to that in the rat reported by Stapleton (Stapleton *et al.*, 1995). The human CYP7B1 gene is about 65kb, which is much larger than CYP7A1 gene (Figure 4.3). The structures of CYP7A1 and CYP7B1 genes are similar. Both contain six exons and five introns and localized very closely on chromosome 8 (Setchell *et al.*, 1998). It is likely that these two genes are derived from ancient gene duplication event. The human promoter and 5'-upstream sequences have been obtained. It contains no TATA box typical for genes expressed in multiple tissues.

### **Sterol 12 $\alpha$ -hydroxylase**

Sterol 12 $\alpha$ -hydroxylase is required for the synthesis of cholic acid (Figure 4.2). The level of expression of this enzyme activity in liver microsomes may play a role in regulating the ratio of cholic acid to chenodeoxycholic acid in different species (Bjorkhem, 1985). The activity is high in rabbit, which produces predominantly cholic acid, and is low in guinea pig, which has CDCA as the major bile acid. Because chenodeoxychoic acid is more hydrophobic and a more potent inhibitor of bile acid synthesis than cholic acid, the ratio of these two primary bile acids in the gallbladder may be important in development of cholesterol gallstones. Bile acids inhibit and cholestyramine stimulates sterol 12 $\alpha$ -hydroxylase activity. The increase of bile acid synthesis, bile acid pool size and the increased ratio of cholic acid to CDCA in diabetes mellitus may be due to the stimulation of 12 $\alpha$ -hydroxylase activity (Kimura *et al.*, 1988). One study revealed that sterol 12 $\alpha$ -hydroxylase activity was increased two-fold in livers of patients treated with cholestyramine or who had undergone ileal resection (Einarsson *et al.*, 1992). The increase of the ratio of CA to CDCA in these patients is likely to be due to a compensatory increase in sterol 12 $\alpha$ -hydroxylase activity. Sterol 12 $\alpha$ -hydroxylase was purified from rabbit liver microsomes (Ishida *et al.*, 1992). Starvation increases the activity several fold in rabbits. Streptozotocin also stimulates enzyme activity. It was reported that this enzyme activity also was inhibited by bile acids in the human liver (Einarsson *et al.*, 1992). The rabbit cDNA encoding sterol 12 $\alpha$ -hydroxylase has been

recently cloned (Eggertsen *et al.*, 1996). It encodes a polypeptide of 500 amino acid residues. The amino acid sequence showed a 39% similarity with human prostacyclin synthase (CYP8) and 31% similarity with rabbit cholesterol 7 $\alpha$ -hydroxylase. This cytochrome P450 gene was designated as CYP8B1. COS cells transfected with CYP8B1 cDNA expressed sterol 12 $\alpha$ -hydroxylase activity toward 7 $\alpha$ -hydroxy-4-cholesten-3-one. Sterol 12 $\alpha$ -hydroxylase metabolized many substrates, including 7 $\alpha$ -hydroxycholesterol, 7 $\alpha$ -hydroxy-4-cholestene-3-one, 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ -diol, 3 $\alpha$ ,7 $\alpha$ -dihydroxy-5 $\beta$ -cholestanoic acid (Andersson *et al.*, 1998). Northern blot analysis showed that the mRNA was exclusively expressed in the liver. Fasting of rats and mice led to a several fold increase in both enzyme activity and mRNA levels. However, starvation of rabbits had little effect on enzyme activity and mRNA levels. Post-translational mechanisms may regulate CYP8B1 gene expression (Eggertsen *et al.*, 1996). The CYP8B1 gene has no intron (Gafvels *et al.*, 1999) (Figure 4.3). This is the first intron-less P450 gene identified.

### 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-sterol dehydrogenase/isomerase

3 $\beta$ -HSD is the second enzyme in the neutral bile acid biosynthesis pathway (Figure 4.2). It catalyzes the oxidation of the 3 $\beta$ -hydroxy group and isomerization of the  $\Delta^5$ -double bond. This enzyme has been purified from pig liver microsomes (Furster *et al.*, 1996). The purified enzyme catalyzed the conversion of 7 $\alpha$ -hydroxycholesterol, 7 $\alpha$ ,25-dihydroxycholesterol, 7 $\alpha$ ,27-dihydroxycholesterol and 3 $\beta$ ,7 $\alpha$ -dihydroxy-5-cholestenoic acid to the corresponding 3-oxo- $\Delta^4$  compounds. The enzyme was inactive toward C<sub>19</sub> and C<sub>21</sub> steroids. Only C<sub>27</sub> steroids with a hydroxyl group in the 7 $\alpha$ -position are substrates for this enzyme. The purified enzyme has an apparent Mr. of 36,000 and is a NAD<sup>+</sup>—dependent dehydrogenase. The N-terminal amino acid sequence of this enzyme is similar to that of 3 $\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenases (Labrie *et al.*, 1992). The cDNA and gene encoding 3 $\beta$ -HSD in bile acid biosynthesis have not been cloned.

## GENETIC DEFECTS IN BILE ACID BIOSYNTHESIS

Primary defects in bile acid synthesis from cholesterol may result in decreased bile formation, which causes malabsorption of vitamins and fats. Defects of enzymes involved in

**Table 4.2** Inborn errors of bile acid biosynthesis.

### A. Primary defects

1. Defective modification of the steroid nucleus: cholesterol and oxysterol 7 $\alpha$ -hydroxylases deficiencies, 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase/isomerase (3 $\beta$ -HSD) deficiency,  $\Delta^4$ - $\Delta^3$ -oxosteroid-5 $\beta$ -reductase deficiency
2. Defective side-chain oxidation: sterol 27-hydroxylase deficiency – Cerebrotendinous xanthomatosis (CTX)

### B. Secondary defects

1. 3 $\beta$ -hydroxysterol  $\Delta^7$ -reductase deficiency – Smith-Lemli-Opitz syndrome
2. Peroxisomal disorders – defects of peroxisome biogenesis and function: cerebrohepato renal (Zellweger) syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, X-linked adrenoleukodystrophy

bile acid biosynthesis cause accumulation of toxic, abnormal bile acid intermediates in the liver, which may interfere with bile acid and xenobiotic transport processes and lead to cholestasis. Primary defects in bile acid biosynthesis include defects in modification of the sterol nucleus (cholesterol and oxysterol 7 $\alpha$ -hydroxylases, 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-sterol oxidoreductase and  $\Delta^{4-3}$ -oxosteroid-5 $\beta$ -reductase) (Table 4.2) and defects in side-chain oxidation (sterol 27-hydroxylase), which has been linked to the cholesterol storage disease CTX (Bjorkhem, 1992, 1994).

Reduction in bile acid synthesis and secretion may be also caused by secondary defects in peroxisomal  $\beta$ -oxidation (Zellweger Syndrome and related diseases) (Balistreri, 1991; Clayton *et al.*, 1988, 1990, 1991) and a defect in *de novo* cholesterol synthesis (Smits-Lemli-Opitz syndrome) (Tint *et al.*, 1994).

## Primary defects

### *Defective modification of the steroid nucleus*

#### *Cholesterol and oxysterol 7 $\alpha$ -hydroxylase deficiency (OMIM entry 118455)*

CYP7A1 is a candidate gene for familial hypertriglyceridemia (Angelin *et al.*, 1978), gallstone disease (Berr *et al.*, 1992; Khanuja *et al.*, 1995; Paumgartner and Sauerbruch, 1991) and hypercholesterolemia (Vega *et al.*, 1987). An inherited deficiency of cholesterol 7 $\alpha$ -hydroxylase has not been described. It is conceivable that a defect in the CYP7A1 gene is lethal and patients will not survive unless they are supplemented with bile acids and vitamins during neonatal life. Cholesterol 7 $\alpha$ -hydroxylase has been shown to influence the expression of the LDL receptor (Dueland *et al.*, 1992) and the assembly and secretion of apoB-containing lipoproteins (Wang *et al.*, 1997). Genetic polymorphisms of the CYP7A1 gene have been identified by single-stranded conformation polymorphism (SSCP) study (Cohen *et al.*, 1992). Four SSCPs in the 5'-flanking region, intron 2 and intron 4 were identified. An *Alu* sequence-related polymorphism was localized in the 3'-flanking region. Of all the alleles analyzed, 80% were heterozygotes for at least one of these five polymorphisms. A *Mae*II polymorphism in the 5'-flanking region was reported (Thompson *et al.*, 1993). A polymorphism of Phe100Ser (TTT to TCT) in human CYP7A1 was identified (Karam and Chiang, 1992). Linkage analysis indicates a significant linkage between CYP7A1 and high plasma LDL-cholesterol concentrations, and not LDL receptor or apolipoprotein B concentration (Wang *et al.*, 1998). Two polymorphisms in the 5'-flanking region (-278C→A and -554C→T) were suggested to contribute to heritable variation in plasma LDL-cholesterol concentrations. These alleles were associated with increased plasma LDL cholesterol concentration, which suggested that these two mutations might reduce the rate of CYP7A1 gene transcription. Genetic linkage analysis of the inbred mice strain C57BL/6J susceptible to atherogenesis has linked multiple loci on chromosomes 3, 5 and 11 to a decrease in HDL-cholesterol after an atherogenic diet. The multiple loci contributing to the decrease of CYP7A1 mRNA levels of B6 mice in response to an atherogenic diet coincided with the loci on chromosomes 3, 5 and 11 controlling HDL levels. Candidate genes at these loci include HNF1 (chromosome 5) and vHNF1 (chromosome 11). HNF1 gene expression is

regulated by HNF4, which regulates the CYP7A1 gene as described above. Interestingly, 3 $\beta$ -HSD genes are also located on chromosomes 3, 5 and 7. These results suggest coordinated regulation of CYP7A1 gene expression and HDL levels (Machleder *et al.*, 1997). Furthermore, two of the loci for CYP7A1 expression may influence the formation of gallstones in strain B6 mice on an atherogenic diet.

Setchell and colleagues (Setchell *et al.*, 1998) recently reported an inborn error of bile acid metabolism due to a defect in oxysterol 7 $\alpha$ -hydroxylase activity. The child in question has severe neonatal cholestasis and cirrhosis. Diagnosis of a defect in 7 $\alpha$ -hydroxylation was revealed by the absence of primary bile acid conjugates and the presence of high levels of 3 $\beta$ -hydroxy- $\Delta^5$ -cholenoic and cholestenic acids in the serum and urine. This points to the block at oxysterol 7 $\alpha$ -hydroxylase, since these two metabolites are formed via the acidic pathway. Both cholesterol 7 $\alpha$ -hydroxylase and oxysterol 7 $\alpha$ -hydroxylase activities were undetectable. This is consistent with the lack of 7 $\alpha$ -hydroxylated bile acids in the patient. No mutation was identified in the CYP7A1 coding exons. Analysis of the CYP7B1 gene identified a C to T mutation in exon 5, which converts Arg388 to a premature termination codon. The mechanism of liver injury in this patient was proposed to be via accumulation of high levels of hepatotoxic monohydroxy bile acids, 24, 25, and 27-hydroxycholesterols. These bile acid metabolites have cholestatic effects by inhibiting bile acid transport across canalicular membranes and reducing bile flow. The lack of cholesterol 7 $\alpha$ -hydroxylase activity in this patient may be due to the accumulation of 3 $\beta$ -hydroxy- $\Delta^5$ -cholenoic and cholestenic acids in the liver, which inhibit cholesterol 7 $\alpha$ -hydroxylase activity, or due to effect of oxysterols on the expression of cholesterol 7 $\alpha$ -hydroxylase at the translational or posttranslational level. It was also suggested that cholesterol 7 $\alpha$ -hydroxylase activity is not expressed in neonatal human liver and bile acid synthesis in early human development proceeds mainly via the acidic pathway (Setchell *et al.*, 1998). Therefore, the lack of both 7 $\alpha$ -hydroxylases in this child caused severe cholestatic liver disease.

*3 $\beta$ - $\Delta^5$ -C<sub>27</sub>-hydroxysteroid dehydrogenase/isomerase (3 $\beta$ -HSD) deficiency*  
(OMIM entry 231100)

Several cases of 3 $\beta$ - $\Delta^5$ -C<sub>27</sub>-hydroxysteroid dehydrogenase/isomerase defects in children have been reported (Buchmann *et al.*, 1990; Clayton *et al.*, 1987). These patients have neonatal giant cell hepatitis and excrete multiple C<sub>24</sub> bile acids with a 3 $\beta$ -hydroxy- $\Delta^5$  structure in their urine. The specific activity of the enzyme was negligible in the patient and low in parents, which was consistent with a heterozygous carrier phenotype (Buchmann *et al.*, 1990). The mechanism of cholestasis in these patients may be linked to the lack of normal bile acids for secretion of bilirubin and other compounds in the bile. The administration of CDCA effectively returned bilirubin to normal levels. CDCA may inhibit cholesterol 7 $\alpha$ -hydroxylase and suppress the synthesis of 3 $\beta$ -hydroxy- $\Delta^5$ -steroids (Ichimiya *et al.*, 1990, 1991). UDCA also has been used for the treatment of these patients (Jacquemin *et al.*, 1994).



*$\Delta^4$ - $^3$ -oxosteroid-5 $\beta$ -reductase deficiency (OMIM entry 235555)*

$\Delta^4$ - $^3$ -oxosteroid-5 $\beta$ -reductase deficiency is another autosomal recessive disorder of bile acid synthesis in a small number of infants with neonatal hepatitis and cholestasis, and liver failure (Setchell *et al.*, 1988, 1994). This enzyme converts 7 $\alpha$ -hydroxy-4-cholesten-3-one and 7 $\alpha$ ,12 $\alpha$ -dihydroxy-4-cholesten-3-one into the corresponding 3-oxo compounds (Table 4.1) (Kondo *et al.*, 1994). The patients excreted taurine conjugated 7 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid and 7 $\alpha$ ,12 $\alpha$ -hydroxy-3-oxo-4-cholenoic acid indicating the defect in  $\Delta^4$ - $^3$ -oxosteroid-5 $\beta$ -reductase.

The pathogenesis of liver disease in this patient may be the result of accumulation of toxic allo-bile salts and a decrease in bile acid-generated bile flow. A significant improvement in the symptoms, laboratory data and normalization of hepatic morphology occurred following feeding of a combination of cholic acid and ursodeoxycholic acid (UDCA). Cholic acid may suppress the synthesis of allo-bile acids via negative feedback control. UDCA is effective in inducing bile flow. Human  $\Delta^4$ - $^3$ -oxosteroid-5 $\beta$ -reductase has been purified and the cDNA cloned (Clayton *et al.*, 1988). However, the molecular basis of the defect has not been identified.

*Defective side-chain oxidation**Sterol 27-hydroxylase deficiency (OMIM entry 213700)*

Deficiency of sterol 27-hydroxylase activity was linked to cerebrotendinous xanthomatosis (CTX), a rare autosomal recessive defect of cholesterol metabolism manifested by tendon xanthomatosis, progressive neurologic dysfunction, accumulation of cholesterol in the tissues, premature atherosclerosis, osteoporosis and cholesterol gallstones (Bjorkhem, 1985). In 1980, Oftebro *et al.* first reported a defect in the mitochondrial 27-hydroxylase activity in CTX patients. The defect in 27-hydroxylase leads to excessive accumulation of 7 $\alpha$ -hydroxycholesterol, 7 $\alpha$ -hydroxy-4-cholesten-3-one, 5 $\beta$ -cholestane-3 $\alpha$ , 7 $\alpha$ ,12 $\alpha$ -triol, cholesterol and cholestanol. The synthesis of bile acids, particularly CDCA, is reduced in CTX patients. This may up-regulate cholesterol 7 $\alpha$ -hydroxylase activity and lead to the accumulation of both 7 $\alpha$ -hydroxycholesterol and 7 $\alpha$ -hydroxy-4-cholesten-3-one. Because bile acid feedback also inhibits HMG-CoA reductase activity, *de novo* cholesterol synthesis may be stimulated in CTX. 7 $\alpha$ -hydroxy-4-cholesten-3-one cannot be metabolized and is converted to cholestanol (Bjorkhem, 1992). Despite normal levels of cholesterol circulation in CTX patients, they develop xanthoma and premature atherosclerosis. This may be due to the reduced elimination of cholesterol from macrophages by sterol 27-hydroxylase.

Most mutations in the CYP27A1 gene of CTX patients are point mutations (Cali *et al.*, 1991; Kim *et al.*, 1994; Garuti *et al.*, 1996; Verrips *et al.*, 1997). Deletion (Leitersdorf *et al.*, 1994; Garuti *et al.*, 1996) and insertion (Segev *et al.*, 1995) mutations were also identified. Most mutations affect the ferredoxin cofactor binding site, the heme-ligand-binding domain (Cali *et al.*, 1991) and the adrenodoxin cofactor binding domain (Chen *et al.*, 1996) and result in non-detectable sterol 27-hydroxylase mRNA and activity (Leitersdorf *et al.*, 1993). These mutations caused frame shift and splicing junction mutations (Leitersdorf *et al.*, 1993; Garuti *et al.*, 1997), alternative pre-mRNA splicing and exon skipping (Garuti *et al.*, 1996; Chen *et al.*, 1996), premature termination (Segev *et al.*,

1995; Garuti *et al.*, 1997) and inframe deletion (Garuti *et al.*, 1997). The prevalence of CTX is high in Japan. CDCA therapy has been used to prevent or reverse neurological symptoms associated with this disease. Early diagnosis of CTX by restriction analysis of three common mutations (Arg441Trp, Arg371Gln, and Arg441Gln) has been reported (Chen *et al.*, 1998).

Despite the link of CYP27A1 gene mutations to CTX, the etiology of this disease is still not known. Disruption of the CYP27A1 gene in mice markedly reduced bile acid synthesis and fecal bile acid excretion by 80% (Rosen *et al.*, 1998). However, no CTX-related phenotypes were observed in CYP27A1<sup>-/-</sup> mice.

## Secondary defects

### *3 $\beta$ -hydroxysterol $\Delta^7$ -reductase deficiency—Smith-Lemli-Opitz syndrome (OMIM entry 270400)*

The Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive disease characterized by microcephaly, poor growth, dysmorphic faces, genital disorders, endocrine malfunction, heart and kidney malformation and mental retardation (Tint *et al.*, 1994). Its prevalence has been estimated to be 1 in 20,000 and may be the second most common autosomal disorder in the North American population, after cystic fibrosis. The extremely low levels of circulating cholesterol are associated with accumulation of 7-dehydrocholesterol. This places the defect at 3 $\beta$ -hydroxysteroid  $\Delta^7$ -reductase, the enzyme converting 7-dehydrocholesterol to cholesterol, which is the last step in the cholesterol synthesis pathway (see also Chapter 2, pp. 49–50). This was shown by markedly inhibited enzyme activity in liver microsomes and fibroblast from SLOS (Shefer *et al.*, 1995). The very low levels of bile acids in patients is likely due to the low levels of cholesterol and also the repression of cholesterol 7 $\alpha$ -hydroxylase activity by accumulated 7-dehydrocholesterol and 27-hydroxycholesterol. A dietary supplement with cholesterol and bile acids may be a useful therapy (Irons *et al.*, 1994). The neurological disorder cannot be reversed, however. 3 $\beta$ -hydroxysterol  $\Delta^7$ -reductase has not been purified and its gene has not been cloned. The SLOS gene has been mapped to 7q32.1 (Alley *et al.*, 1997). The molecular basis of Smith-Lemli-Opitz syndrome has not been confirmed.

### *Peroxisomal disorders (OMIM entry 214100)*

Peroxisomal  $\beta$ -oxidation enzymes are required for sterol side-chain oxidation in bile acid biosynthesis. 3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -trihydroxy-5 $\beta$ -cholestanoic acid (THCA) and 3 $\alpha$ , 7 $\alpha$ -dihydroxy-5 $\beta$ -cholestanoic acid (DHCA) are converted to cholic acid and chenodeoxycholic acid, respectively, by peroxisomal  $\beta$ -oxidation enzymes. A generalized impairment of peroxisome biogenesis causes Zellweger and related syndromes, infantile Refsum disease, neonatal adrenoleukodystrophy and X-linked adrenoleukodystrophy (Lazarow and Moser, 1989). The Zellweger syndrome (or cerebrohepato renal syndrome) is a congenital syndrome of multiple manifestations, including hepatomegaly and liver dysfunction. Peroxisomes are absent in the liver and kidney of Zellweger patients (Goldfisher *et al.*, 1997) and THCA and DHCA accumulate (Hanson *et al.*, 1979). Treatment is aimed at improving nutrition and growth, controlling central nervous system symptoms and limiting progression of liver disease. The liver disease in Zellweger syndrome may be attributed to an overproduction and accumulation of cholestanoic acids, exacerbated by

diminished primary bile acid synthesis. Primary bile acid administration was beneficial in improving liver function by down-regulation of the synthesis of these atypical bile acids (Setchell *et al.*, 1992).

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### REFERENCES

- Alley, T.L., Scherer, S.W., Huizenga, J.J., Tsui, L.C. and Wallace, M.R. (1997) Physical mapping of the chromosome 7 breakpoint region in an SLOS patient with t(7;20) (q32.1;q13.2). *Am. J. Med. Genet.* **68**, 279–281.
- Andersson, S., Davis, D.L., Dahlback, H., Jornvall, H. and Russell, D.W. (1989) Cloning, structure, and expression of the mitochondrial cytochrome P450 sterol 27-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264**, 8222–8229.
- Andersson, U., Eggertsen, G. and Bjorkhem, I. (1998) Rabbit liver contains one major sterol 12 $\alpha$ -hydroxylase with broad substrate specificity. *Biochim. Biophys. Acta* **1389**, 150–154.
- Angelin, B., Einarsson, K., Hellstrom, K. and Leijdt, B. (1978) Bile acid kinetics in relation to endogenous triglyceride metabolism in various types of hyperlipoproteinemia. *J. Lipid Res.* **19**, 1004–1016.
- Araya, Z., Sjoberg, H. and Wikvall, K. (1995) Different effects on the expression of CYP7 and CYP27 in rabbit liver by cholic acid and cholestyramine. *Biochem. Biophys. Res. Commun.* **216**, 868–873.
- Arnon, R., Yoshimura, T., Reiss, A., Budai, K., Lefkowitz, J.H. and Javitt, N.B. (1998) Cholesterol 7 $\alpha$ -hydroxylase knockout mouse: A model for monohydroxy bile acid-related neonatal cholestasis. *Gastroenterology* **115**, 1223–1228.
- Axelson, M., Shoda, J., Sjoval, J., Toll, A. and Wikvall, K. (1992) Cholesterol is converted to 7 $\alpha$ -hydroxy-3-oxo-4-cholestenic acid in liver mitochondria. Evidence for a mitochondrial sterol 7 $\alpha$ -hydroxylase. *J. Biol. Chem.* **267**, 1701–1704.
- Axeison, M. and Sjoval, J. (1990) Potential bile acid precursors in plasma-possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. *J. Steroid Biochem.* **36**, 631–640.
- Babiker, A., Andersson, O., Lund, E., Xiu, R.-J., Deeb, S., Reshef, A., Leitersdorf, E., Diczfalussy, U. and Bjorkhem, I. (1997) Elimination of cholesterol in macrophages and endothelial cells by sterol 27-hydroxylase mechanism. *J. Biol. Chem.* **272**, 26253–26261.
- Balistreri, W.F. (1991) Fetal and neonatal bile acid synthesis and metabolism—clinical implications. *J. Inher. Metab. Dis.* **14**, 459–77.
- Balistreri, W.F. (1995) Inborn errors of bile acid biosynthesis: clinical and therapeutic aspects. In: *Bile acids in Gastroenterology: Basic and Clinical Advances*, A.F. Hofmann, G. Paumgartner and A. Stiehl (eds), Kluwer Acad. Pub., Dordrecht, pp. 333–353.
- Berkowitz, C.M., Shen, C.S., Bilir, B.M., Guibert, E. and Gumucio, J.J. (1995) Different hepatocytes express the cholesterol 7 $\alpha$ -hydroxylase gene during its circadian modulation *in vivo*. *Hepatology* **21**, 1658–1667.
- Berr, F., Pratschke, E., Fisher, S. and Paumgartner, G. (1992) Disorders of bile acid metabolism in cholesterol gallstone disease. *J. Clin. Invest.* **90**, 859–868.

- Bertolotti, M., Abate, N., Loria, P., Dilengite, M., Carubbi, F., Pinetti, A., Digrisolo, A. and Carulli, N. (1991) Regulation of bile acid synthesis in humans: Effect of treatment with bile acids, cholestyramine, or simvastatin on cholesterol 7 $\alpha$ -hydroxylation rates in vivo. *Hepatology* **14**, 830–837.
- Bertolotti, M., Concarì, M., Loria, P., Agate, N., Pinetti, A., Guicciardi, M.E. and Carulli, N. (1995) Effects of different phenotypes of hyperlipoproteinemia and of treatment with fibric acid derivatives on the rates of cholesterol 7 $\alpha$ -hydroxylation in humans. *Arterio. Thromb. Vasc. Biol.* **15**, 1064–1069.
- Bjorkhem, I. (1994) Inborn errors of metabolism with consequences for bile acid biosynthesis. A minireview. *Scand. J. Gastroenterol.* **204** (Suppl), 68–72.
- Bjorkhem, I. (1985) Mechanism of bile acid biosynthesis in mammalian liver. In: *Steroid and bile acid*, Danielsson, H. and Sjovall, J. (eds), The Netherlands: Elsevier Sci. Pub. pp. 231–277.
- Bjorkhem, I. (1992) Mechanism of degradation of the steroid side chain in the formation of bile acids. *J. Lipid Res.* **33**, 455–471.
- Bjorkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R.-J., Duan, C. and Lund, E. (1994) Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8592–8596.
- Bjorkhem, I., Nyberg, B. and Einarsson, K. (1992) 7 $\alpha$ -hydroxylation of 27-hydroxycholesterol in human liver microsomes. *Biochim. Biophys. Acta* **1128**, 73–76.
- Bocos, C., Gottlicher, M., Gearing, K., Banner, C., Enmark, E., Teboul, M., Crickmore, A. and Gustafsson, J. (1995) Fatty acid activation of peroxisome proliferator-activated receptor (PPAR). *J. Steroid Biochem. Molec. Biol.* **53**, 467–473.
- Buchmann, M.S., Kvittingen, E.A., Nazer, H., Gunasekaran, T., Clayton, P.T., Sjovall, J. and Bjorkhem, I. (1990) Lack of 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase/isomerase in fibroblasts from a child with urinary excretion of 3 $\beta$ -hydroxy- $\Delta^5$ -bile acids. A new inborn error of metabolism. *J. Clin. Invest.* **86**, 2034–2037.
- Brown, A.J. and Jessup, W. (1999) Oxysterols and atherosclerosis. *Atherosclerosis* **142**, 1–28.
- Cali, J.J., Hsieh, C.-L., Francke, U. and Russell, D.W. (1991) Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. *J. Biol. Chem.* **266**, 7779–7783.
- Cali, J.J. and Russell, D.W. (1991) Characterization of human sterol 27-hydroxylase. *J. Biol. Chem.* **266**, 7774–7778.
- Chen, W., Kubota, S., Nishimura, Y., Nozaki, S., Yamashita, S., Nakagawa, T., Kameda-Takemura, K., Menju, M., Matsuzawa, Y., Bjorkhem, I., Eggertsen, G. and Seyama, Y. (1996) Genetic analysis of a Japanese cerebrotendinous xanthomatosis family: identification of a novel mutation in the adrenodoxin binding region of the CYP27 gene. *Biochim. Biophys. Acta* **1317**, 119–126.
- Chen, W., Kubota, S., Ujike, H., Ishihara, T. and Seyama, Y. (1998) A novel Arg362Ser mutation in the sterol 27-hydroxylase gene (CYP27): its effects on pre-mRNA splicing and enzyme activity. *Biochemistry* **37**, 15050–15056.
- Chiang, J.Y.L. (1998) Regulation of bile acid synthesis. *Frontier in Biosciences* **3**, D176–D193.
- Chiang, J.Y.L., Miller, W.F. and Lin, G.M. (1990) Regulation of cholesterol 7 $\alpha$ -hydroxylase in the liver: purification of cholesterol 7 $\alpha$ -hydroxylase and the immunochemical evidence for the induction of cholesterol 7 $\alpha$ -hydroxylase by cholestyramine and circadian rhythm. *J. Biol. Chem.* **265**, 3889–3897.
- Chiang, J.Y.L. and Stroup, D. (1994) Identification and characterization of a putative bile acid responsive element in cholesterol 7 $\alpha$ -hydroxylase gene promoter. *J. Biol. Chem.* **269**, 17502–17507.

- Chiang, J.Y.L. and Vlahcevic, Z.R. (1996) The Regulation of Cholesterol Conversion to Bile Acids. In: *Advances in Molecular and Cellular Biology*, Jefcoate, C. (ed), JAI Press, Inc., London, pp. 269–316.
- Chiang, J.Y.L., Yang, T.P. and Wang, D.P. (1992) Cloning and 5'-flanking sequence of a rat cholesterol 7 $\alpha$ -hydroxylase gene. *Biochim. Biophys. Acta* **1132**, 337–339.
- Clayton, P.T. (1991) Inborn errors of bile acid metabolism. *J. Inherit. Metab. Dis.* **14**, 478–496.
- Clayton, P.T., Lake, B.D., Hjelm, M., Stephenson, J.B., Besley, G.T., Wanders, R.J., Schram, A.W., Tager, J.M., Schutgens, R.B. and Lawson, A.M. (1988) Bile acid analyses in "pseudo-Zellweger" syndrome; clues to the defect in peroxisomal  $\beta$ -oxidation. *J. Inherit. Metab. Dis.* **11**, 165–168.
- Clayton, P.T., Leonard, J.V., Lawson, A.M., Setchell, K.D., Andersson, S., Egestad, B. and Sjoval, J. (1987) Familial giant cell hepatitis associated with synthesis of 3 $\beta$ ,7 $\alpha$ -dihydroxy- and 3 $\beta$ ,7 $\alpha$ ,12 $\alpha$ -trihydroxy-5-choleenoic acids. *J. Clin. Invest.* **79**, 1031–1038.
- Clayton, P.T., Patel, E., Lawson, A.M., Carruthers, R.A. and Collins, J. (1990) Bile acid profiles in peroxisomal 3-oxoacyl-coenzyme A thiolase deficiency. *J. Clin. Invest.* **85**, 1267–1273.
- Clayton, P.T., Patel, E., Lawson, A.M., Carruthers, R.A., Tanner, M.S., Strandvik, B., Egestad, B. and Sjoval, J. (1988) 3-Oxo- $\Delta^4$  bile acids in liver disease. *Lancet* **1**, 1283–1284.
- Cohen, J.C., Cali, J.J., Jelinek, D.F., Mehrabian, M., Sparkes, R.S., Lusi, A.J., Russell, D.W., and Hobbs, H.H. (1992) Cloning of the human cholesterol 7 $\alpha$ -hydroxylase gene (CYP7) and localization to chromosome 8q11-q12. *Genomics* **14**, 153–161.
- Cooney, A.J., Tsai, S.Y., O'Malley, B.W. and Tsai, M.-J. (1992) Chicken Ovalbumin Upstream Promoter transcription factor (COUP-TF) dimers bind to different GGTCa response elements, allowing COUP-TF to repress hormonal induction of the vitamin D<sub>3</sub>, thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.* **12**, 4153–4163.
- Cooper, A.D., Chen, J., Botelho-Yetkinler, M.J., Cao, Y., Taniguchi, T. and Levy-Wilson, B. (1997) Characterization of hepatic-specific regulatory elements in the promoterregion of the human cholesterol 7 $\alpha$ -hydroxylase gene. *J. Biol. Chem.* **272**, 3444–3452.
- Crestani, M., Galli, G. and Chiang, J.Y.L. (1993) Genomic cloning, sequencing and analysis of hamster cholesterol 7 $\alpha$ -hydroxylase gene (CYP7). *Arch. Biochem. Biophys.* **306**, 451–460.
- Crestani, M., Sadeghpour, A., Stroup, D., Galli, G. and Chiang, J.Y.L. (1996) The opposing effects of retinoic acid and phorbol esters converge to a common response element in the promoter of the rat cholesterol 7 $\alpha$ -hydroxylase gene (CYP7A). *Biochem. Biophys. Res. Commun.* **225**, 585–592.
- Crestani, M., Sadeghpour, A., Stroup, D., Gali, G. and Chiang, J.Y.L. (1998) Transcriptional activation of the cholesterol 7 $\alpha$ -hydroxylase gene (CYP7A) by nuclear hormone receptors. *J. Lipid Res.* **39**, 2192–2200.
- Crestani, M., Stroup, D., and Chiang, J.Y.L. (1995) Hormonal regulation of the cholesterol 7 $\alpha$ -hydroxylase gene (CYP7). *J. Lipid Res.* **36**, 2419–2432.
- Crisby, M., Nilsson, J., Kostulas, V., Bjorkhem, I. and Diczfalussy, U. (1997) Localization of sterol 27-hydroxylase immuno-reactivity in human atherosclerotic plaques. *Biochim. Biophys. Acta* **1344**, 278–285.
- Dahlback-Sjoberg, H., Bjorkhem, I. and Princen, H.M.G. (1993) Selective inhibition of mitochondrial 27-hydroxylation of bile acid intermediates and 25-hydroxylation of vitamin D<sub>3</sub> by cyclosporin A. *Biochem. J.* **293**, 203–206.
- Dawson, P.A. and Oelkers, P. (1995) Bile acid transporters. *Curr. Opin. Lipidol.* **6**, 109–114.
- Del Puppo, M., Kienle, M.G., Petroni, M.L., Crosignani, A. and Podda, M. (1998) Serum 27-hydroxycholesterol in patients with primary biliary cirrhosis suggests alteration of cholesterol catabolism to bile acids via the acidic pathway. *J. Lipid Res.* **39**, 2477–2482.

- Devchand, P.R., Keller, H., Peters, J.M., Vazquez, M., Gonzalez, F.J. and Wahli, W. (1996) The PPAR $\alpha$ -leukotriene B $_4$  pathway to inflammation control. *Nature* **384**, 39–43.
- Doerner, K.C., Gurley, E.G., Vlahcevic, Z.R. and Hylemon, P.B. (1995) Regulation of cholesterol 7 $\alpha$ -hydroxylase expression by sterols in primary hepatocyte cultures. *J. Lipid Res.* **36**, 168–177.
- Dueland, S., Drisko, J., Graf, L., Machleder, D., Lusis, A.J. and Davis, R.A. (1993) Effect of dietary cholesterol and taurocholate on cholesterol 7 $\alpha$ -hydroxylase and hepatic LDL receptors in inbred mice. *J. Lipid Res.* **34**, 923–931.
- Dueland, S., Trawick, J.D., Nenseter, M.S., MacPhee, A.A. and Davis, R.A. (1992) Expression of 7 $\alpha$ -hydroxylase in non-hepatic cells results in liver phenotypic resistance of the low density lipoprotein receptor to cholesterol repression. *J. Biol. Chem.* **267**, 22695–22698.
- Eggertsen, G., Olin, M., Andersson, U., Ishida, H., Kubota, S., Hellman, U., Okuda, K.-I. and Bjorkhem, I. (1996) Molecular cloning and expression of rabbit sterol 12 $\alpha$ -hydroxylase. *J. Biol. Chem.* **271**, 32269–32275.
- Einarsson, K., Akerlund, J.-E., Reihner, E. and Bjorkhem, I. (1992) 12 $\alpha$ -Hydroxylase activity in human liver and its relation to cholesterol 7 $\alpha$ -hydroxylase activity. *J. Lipid Res.* **33**, 1591–1595.
- Falvey, E., Fleury-Olela, F. and Schibler, U. (1995) The rat hepatic leukemia factor (HLF) gene encodes two transcriptional activators with distinct circadian rhythms, tissue distributions and target preferences. *EMBO J.* **14**, 4307–4317.
- Forman, B.M., Chen, J. and Evans, R.M. (1997a) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors  $\alpha$  and  $\delta$ . *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4312–4317.
- Forman, B.M., Ruan, B., Chen, J., Schroepfer, G.J. and Evans, R.M. (1997b) The orphan nuclear receptor LXR $\alpha$  is positively and negatively regulated by distinct products of mevalonate metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 10588–10593.
- Foti, D., Stroup, D. and Chiang, J.Y. (1998) Basic transcription element binding protein (BTEB) transactivates the cholesterol 7 $\alpha$ -hydroxylase gene (CYP7A). *Biochem. Biophys. Res. Commun.* **253**, 109–113.
- Furster, C., Zhang, J. and Toll, A. (1996) Purification of a 3 $\beta$ -hydroxy- $\Delta^5$ -C $_{27}$ -sterol dehydrogenase from pig liver microsomes active in major and alternative pathways of bile acid biosynthesis. *J. Biol. Chem.* **271**, 20903–20907.
- Gafvels, M., Olin, M., Chowdhary, B.P., Raudsepp, T., Andersson, U., Persson, B., Jansson, M., Bjorkhem, I. and Eggertsen, G. (1999) Structure and chromosomal assignment of the sterol 12 $\alpha$ -hydroxylase gene (CYP8B1) in human and mouse: eukaryotic cytochrome P450 gene devoid of introns. *Genomics*, **56**, 184–196.
- Garuti, R., Croce, M.A., Tiozzo, R., Dotti, M.T., Federico, A., Bertolini, S. and Calandra, S. (1997) Four novel mutations of sterol 27-hydroxylase gene in Italian patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* **38**, 2322–2334.
- Garuti, R., Lelli, N., Barozzini, M., Dotti, M.T., Federico, A., Bertolini, S. and Calandra, S. (1996) Partial deletion of the gene encoding sterol 27-hydroxylase in a subject with cerebrotendinous xanthomatosis. *J. Lipid Res.* **37**, 662–672.
- Ginsburg, G.S., Ozer, J. and Karathanasis, S.K. (1995) Intestinal apolipoprotein A1 gene transcription is regulated by multiple distinct DNA elements and is synergistically activated by the orphan nuclear receptor, hepatocyte nuclear factor 4. *J. Clin. Invest.* **96**, 528–538.
- Goldfisher, S., Moore, C.L., Johnson, A.B., Spiro, A.I., Valsamis, M.P., Wisniewski, H.K., Ritch, R.H., Norton, W.T., Rapin, I. and Gartner, M. (1997) Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. *Science* **182**, 62–64.

- Guo, Y.-D., Strugnell, S., Back, D.W. and Jones, G. (1993) Transfected human liver cytochrome P-450 hydroxylase vitamin D analogs at different side-chain positions. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8668–8672.
- Hall, R.K., Sladek, P.M. and Granner, D.K. (1995) The orphan receptors COUP-TF and HNF-4 serve as accessory factors required for induction of phosphoenolpyruvate carboxykinase gene transcription by glucocorticoids. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 412–416.
- Hanson, R.F., Szczepanik-Van Leeuwen, P., Williams, G.C., Grabowski, G. and Sharp, H.L. (1979) Defects of bile acid synthesis in Zellweger's syndrome. *Science*, **203**, 1107–1108.
- Hertz, R., Magenheimer, J., Berman, I. and Bar-Tana, J. (1998) Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4a. *Nature* **392**, 512–516.
- Heuman, D.M., Hylemon, P.B. and Vlahcevic, Z.R. (1989) Regulation of bile acid synthesis. III Correlation between biliary bile salt hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat. *J. Lipid Res.* **30**, 1160–1171.
- Hoekman, M.F.M., Rientjes, J.M.J., Twisk, J., Planta, R.J., Princen, H.M.G. and Mager, W.H. (1993) Transcriptional regulation of the gene encoding cholesterol 7 $\alpha$ -hydroxylase in the rat. *Gene* **130**, 217–223.
- Horton, J.D., Cuthbert, J.A. and Spady, D.K. (1995) Regulation of hepatic 7 $\alpha$ -hydroxylase expression and response to dietary cholesterol in the rat and hamster. *J. Biol. Chem.* **270**, 5381–5387.
- Hylemon, P.B., Gurley, E.G., Stravitz, R.T., Litz, J.S., Pandak, W.M., Chiang, J.Y.L. and Vlahcevic, Z.R. (1992) Hormonal regulation of cholesterol 7 $\alpha$ -hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. *J. Biol. Chem.* **267**, 16866–16871.
- Ichimiya, H., Egestad, B., Nazer, H., Baginski, E.S., Clayton, P.T. and Sjovall, J. (1991) Bile acids and bile alcohols in a child with hepatic 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency: effects of chenodeoxycholic acid treatment. *J. Lipid Res.* **32**, 829–841.
- Ichimiya, H., Nazer, H., Gunasekaran, T., Clayton, P. and Sjovall, J. (1990) Treatment of chronic liver disease caused by 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency with chenodeoxycholic acid. *Arch. Dis. Child* **65**, 1121–1124.
- Imataka, H., Sogawa, K., Yasumoto, K.-L., Kikuchi, Y., Sasano, K., Kobayashi, A., Hayami, M. and Fujii-Kuriyama, Y. (1992) Two regulatory proteins that bind to the basic transcriptional element (BTE), a GC box sequence in the promoter region of the rat P-4501A1 gene. *EMBO J.* **11**, 3663–3671.
- Irons, M., Elias, E.R., Tint, G.S., Salen, G., Frieden, R., Buie, T.M. and Ampola, M. (1994) Abnormal cholesterol metabolism in the Smith-Lemli-Opitz syndrome: report of clinical and biochemical findings in four patients and treatment in one patient. *Am. J. Med. Genet.* **50**, 347–352.
- Ishibashi, S., Schwartz, M., Frykman, P.K., Hertz, J. and Russell, D.W. (1996) Disruption of cholesterol 7 $\alpha$ -hydroxylase gene in mice: I. Postnatal lethality reversed by bile acid and vitamin supplementation. *J. Biol. Chem.* **271**, 18017–18023.
- Ishida, H., Noshiro, M., Okuda, K. and Coon, M.J. (1992) Purification and characterization of 7 $\alpha$ -hydroxy-4-cholesten-3-one 12 $\alpha$ -hydroxylase. *J. Biol. Chem.* **267**, 21319–21323.
- Jacquemin, E., Setchell, K.D., O'Connell, N.C., Estrada, A., Maggiore, G., Schmitz, J., Hadchouel, M. and Bernard, O. (1994) A new cause of progressive intrahepatic cholestasis: 3 $\beta$ -hydroxy-C<sup>27</sup>-steroid dehydrogenase/isomerase deficiency. *J. Pediatr.* **125**, 379–384.
- Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R. and Mangelsdorf, D.J. (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR $\alpha$ . *Nature* **383**, 728–731.
- Javitt, N.B. (1994) Bile acid synthesis from cholesterol: regulatory and auxiliary pathways. *FASEB J.* **8**, 1308–1311.

- Javitt, N.B., Pfeffer, R., Kok, E., Burstein, S., Cohen, B.I. and Budai, K. (1989) Bile acid synthesis in cell culture. *J. Biol. Chem.* **264**, 10384–10387.
- Jelinek, D.F., Andersson, S., Slaughter, C.A. and Russell, D.W. (1990) Cloning and regulation of cholesterol 7 $\alpha$ -hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. *J. Biol. Chem.* **265**, 8190–8197.
- Jelinek, D. and Russell, D.W. (1990) Structure of the rat gene encoding cholesterol 7 $\alpha$ -hydroxylase. *Biochemistry* **29**, 7781–7785.
- Jiang, G., Nepomuceno, L., Hopkins, K. and Sladek, P.M. (1995) Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. *Mol. Cell. Biol.* **15**, 5131–5143.
- Jones, M.P., Pandak, W.M., Hylemon, P.B., Chiang, J.Y.L., Heuman, D.M. and Vlahcevic, Z.R. (1993) Cholesterol 7 $\alpha$ -hydroxylase: Evidence for transcriptional regulation by cholesterol and/or metabolic products of cholesterol in the rat. *J. Lipid Res.* **34**, 885–892.
- Karam, W.G. and Chiang, J.Y.L. (1992) Polymorphisms of human cholesterol 7 $\alpha$ -hydroxylase. *Biochem. Biophys. Res. Commun.* **185**, 588–595.
- Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. and Wahli, W. (1993) Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2160–2164.
- Kern, F. (1991) Normal plasma cholesterol in an 88-year-old man who eats 25 eggs a day. *N. Engl. J. Med.* **324**, 896–899.
- Khanuja, B., Cheah, Y.-C., Hunt, M., Nishina, P.M., Wang, D.Q.-H., Chen, H.W., Billheimer, J.T., Carey, M.C. and Paigen, B. (1995) *Lith 1*, a major gene affecting cholesterol gallstone formation among inbred strains of mice. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7729–7733.
- Kim, K.-S., Kubota, S., Kuriyama, M., Fujiyama, J., Bjorkhem, I., Eggertsen, G. and Seyama, Y. (1994) Identification of new mutations in sterol 27-hydroxylase gene in Japanese patients with cerebrotendinous xanthomatosis (CTX). *J. Lipid Res.* **35**, 1031–1039.
- Kimura, A., Nishiyori, A., Murakami, T., Tsukamoto, T., Hata, S., Osumi, T., Okamura, R., Mori, M. and Takiguchi, M. (1993) Chicken Ovalbumin Upstream Promoter-Transcription Factor (COUP-TF) represses transcription from the promoter of the gene for ornithine transcarbamylase in a manner antagonistic to hepatocyte nuclear factor-4 (HNF-4). *J. Biol. Chem.* **268**, 11125–11133.
- Kimura, K., Ogura, Y. and Ogura, M. (1988) Increased rate of cholic acid formation from 3 $\alpha$ ,7 $\alpha$ -dihydroxy-5 $\beta$ -cholestane in perfused livers from diabetic rats. *Biochim. Biophys. Acta* **963**, 329–332.
- Kondo, K.H., Kai, M.H., Setoguchi, Y., Eggertsen, G., Sjoblom, P., Setoguchi, T., Okuda, K.I. and Bjorkhem, I. (1994) Cloning and expression of cDNA of human  $\Delta^4$ -3-oxosteroid 5 $\beta$ -reductase and substrate specificity of the expressed enzyme. *Eur. J. Biochem.* **219**, 357–363.
- Kozarsky, K.F., Donahee, M.H., Rigotti, A., Iqbal, S.N., Edelman, E.R. and Krieger, M. (1997) Over-expression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. *Nature* **387**, 414–417.
- Ktistaki, E. and Talianidis, I. (1997) Chicken ovalbumin upstream promoter transcription factors act as auxiliary cofactors for hepatocyte nuclear factor 4 and enhance hepatic gene expression. *Mol. Cell. Biol.* **17**, 2790–2797.
- Labrie, F., Simard, J., Luu-The, V., Pelletier, G., Belanger, A., Lachance, Y., Zhao, H.F., Labrie, C., Breton, N., de Launoit, Y., *et al.* (1992). Structure and tissue-specific expression of 3 $\beta$ -hydroxysteroid dehydrogenase/5-ene-4-ene isomerase genes in human and rat classical and peripheral steroidogenic tissues. *J. Steroid Biochem. Molec. Biol.* **41**, 421–435.



- Lavery, D.J. and Schibler, U. (1993) Circadian transcription of the cholesterol 7 $\alpha$ -hydroxylase gene may involve the liver-enriched bZIP protein DBP. *Genes Devel.* **7**, 1871–1884.
- Lazarow, P.B. and Moser, H.W. (1989) In: *Disorder of peroxisomes biogenesis*, 6th Edition, C.R.Scriver, A.L.Beaudet, W.S.Sly and D.Valle (eds) McGraw Hill, New York.
- Lee, Y.-H., Alberta, J.A., Gonzalez, F.J. and Waxman, D.J. (1994) Multiple, functional DBP sites on the promoter of the cholesterol 7 $\alpha$ -hydroxylase P450 gene, CYP7. *J. Biol. Chem.* **269**, 14681–14689.
- Lehmann, J.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.-L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A. and Wilson, T.M. (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J. Biol. Chem.* **272**, 3137–3140.
- Leitersdorf, E., Reshef, A., Meiner, V., Levitzki, R., Schwartz, S.P., Dann, E.J., Berkman, N., Cali, J.L., Klapholz, L. and Berginer, V.M. (1993) Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous Xanthomatosis in Jews of Moroccan origin. *J. Clin. Invest.* **91**, 2488–2496.
- Leitersdorf, E., Safadi, R., Meiner, V., Reshef, A., Bjorkhem, I., Friedlander, Y., Morkos, S. and Berginer, V.M. (1994) Cerebrotendinous xanthomatosis in the Israeli Druze: molecular genetics and phenotypic characteristics. *Am. J. Hum. Genet.* **55**, 907–915.
- Li, Y.C., Wang, D.P. and Chiang, J.Y.L. (1990) Regulation of cholesterol 7 $\alpha$ -hydroxylase in the liver: cDNA cloning, sequencing and regulation of cholesterol 7 $\alpha$ -hydroxylase mRNA. *J. Biol. Chem.* **265**, 12012–12019.
- Lund, E., Bjorkhem, I., Furster, C. and Wikvall, K. (1993) 24, 25- and 27-hydroxylation of cholesterol by a purified preparation of 27-hydroxylase from pig liver. *Biochim. Biophys. Acta* **1166**, 177–182.
- Lund, E.G., Kerr, T.A., Sakai, J., Li, W.-P. and Russell, D.W. (1998) cDNA cloning of mouse and human cholesterol 25-hydroxylases, polytopic membrane proteins that synthesizes a potent oxysterol regulators of lipid metabolism. *J. Biol. Chem.* **273**, 34316–34327.
- Machleder, D., Ivandic, B., Welch, C., Castellani, L., Reue, K. and Lusis, A.J. (1997) Complex genetic control of HDL levels in mice in response to an atherogenic diet. *J. Clin. Invest.* **99**, 1406–1419.
- Martin, K.O., Budai, K. and Javitt, N.B. (1993) Cholesterol and 27-hydroxycholesterol 7 $\alpha$ -hydroxylation: evidence for two different enzymes. *J. Lipid Res.* **34**, 581–588.
- Martin, K.O., Reiss, A.B., Lathe, R. and Javitt, N.B. (1997) 7 $\alpha$ -hydroxylation of 27-hydroxycholesterol: biologic role in the regulation of cholesterol synthesis. *J. Lipid Res.* **38**, 1053–1058.
- Molowa, D.T., Chen, W.S., Cimis, G.M. and Tan, C.P. (1992) Transcriptional regulation of the human cholesterol 7 $\alpha$ -hydroxylase gene. *Biochemistry* **31**, 2539–2544.
- Myant, N.B. and Mitropoulos, K.A. (1977). Cholesterol 7 $\alpha$ -hydroxylase. *J. Lipid Res.* **18**, 135–153.
- Ness, G.C., Pendleton, L.C., Li, Y.C. and Chiang, J.Y.L. (1990) Effect of thyroid hormone on hepatic cholesterol 7 $\alpha$ -hydroxylase, LDL receptor, HMG-CoA reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-I mRNA levels in hypophysectomized rats. *Biochem. Biophys. Res. Commun.* **172**, 1150–1156.
- Ness, G.C., Pendleton, L.C. and Zhao, Z. (1994a) Thyroid hormone rapidly increases cholesterol 7 $\alpha$ -hydroxylase mRNA levels in hypophysectomized rats. *Biochim. Biophys. Acta* **1214**, 229–233.
- Ness, G.C., Zhao, Z. and Keller, R.K. (1994b) Effect of squalene synthase inhibition on the expression of hepatic cholesterol biosynthetic enzyme, LDL receptor, and cholesterol 7 $\alpha$ -hydroxylase. *Arch. Biochem. Biophys.* **311**, 277–285.

- Norlin, M. and Wikvall, K. (1998) Biochemical characterization of the 7 $\alpha$ -hydroxylase activities toward 27-hydroxycholesterol and dehydroepiandrosterone in pig liver microsomes. *Biochim. Biophys. Acta* **1390**, 269–281.
- Noshiro, M., Nishimoto, M., Morohashi, K. and Okuda, K. (1989) Molecular cloning of cDNA for cholesterol 7 $\alpha$ -hydroxylase from rat liver microsomes: nucleotide sequence and expression. *FEBS Lett.* **257**, 97–100.
- Noshiro, M., Nishimoto, M. and Okuda, K. (1990) Rat liver cholesterol 7 $\alpha$ -hydroxylase. Pretranslational regulation for circadian rhythm. *J. Biol. Chem.* **265**, 10036–10041.
- Oftebro, H., Bjorkhem, I., Skrede, S., Schreiner, A. and Pederson, J.I. (1980) Cerebrotendinous xanthomatosis: a defect in mitochondrial 26-hydroxylation required for normal biosynthesis of cholic acid. *J. Clin. Invest.* **65**, 1418–1430.
- Pandak, W.M., Heuman, D.M., Redford, K., Stravitz, R.T., Chiang, J.Y.L., Hylemon, P.B. and Vlahcevic, Z.R. (1997) Hormonal regulation of cholesterol 7 $\alpha$ -hydroxylase specific activity, mRNA levels and transcriptional activity *in vivo* in the rat. *J. Lipid Res.* **38**, 2483–2491.
- Pandak, W.M., Vlahcevic, Z.R., Chiang, J.Y.L., Heuman, D.M. and Hylemon, P.B. (1992) Bile acid synthesis: VI Regulation of cholesterol 7 $\alpha$ -hydroxylase by taurocholate and mevalonate. *J. Lipid Res.* **33**, 659–668.
- Pandak, W.M., Vlahcevic, Z.R., Heuman, D.M. and Hylemon, P.B. (1990) Regulation of bile acid synthesis: V. Inhibition of conversion of 7-dehydrocholesterol to cholesterol is associated with down-regulation of cholesterol 7 $\alpha$ -hydroxylase activity and inhibition of bile acid synthesis. *J. Lipid Res.* **31**, 2149–2158.
- Pamgartner, G. and Sauerbruch, T. (1991) Gallstones: pathogenesis. *Lancet* **338**, 1117–1121.
- Payne, D.W., Shackleton, C., Toms, H., Ben-Shlomo, I., Kol, S., deMoura, M., Strauss, J.F. and Adashi, E. (1995) A novel nonhepatic hydroxycholesterol 7 $\alpha$ -hydroxylase that is markedly stimulated by interleukin-1 $\beta$ . *J. Biol. Chem.* **270**, 18888–18896.
- Peet, D.J., Janowski, B.A. and Mangelsdorf, D.J. (1998a) The LXRs: a new class of oxysterol receptors. *Curr. Opin. Genet. Devel.* **8**, 571–575.
- Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.M., Hammer, R.E. and Mangelsdorf, D.J. (1998b) Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR $\alpha$ . *Cell* **93**, 693–704.
- Pikuleva, I.A., Babiker, A., Waterman, M.R. and Björkhem, I. (1998) Activities of recombinant human cytochrome P450c27 (CYP27) which produce intermediates of alternative bile acid biosynthetic pathways. *J. Biol. Chem.* **273**, 18153–18160.
- Pikuleva, I.A., Bjorkhem, I. and Waterman, M.R. (1997) Expression, purification and enzymatic properties of recombinant human cytochrome P450c27 (CYP27). *Arch. Biochem. Biophys.* **343**, 123–130.
- Poorman, J.A., Buck, R.A., Smith, S.A., Overturf, M.L. and Loose-Mitchell, D.S. (1993) Bile acid excretion and cholesterol 7 $\alpha$ -hydroxylase expression in hypercholesterolemia-resistant rabbits. *J. Lipid Res.* **34**, 1675–1685.
- Princen, H.M.G., Meijer, P. and Hofstee, B. (1989) Dexamethasone regulates bile acid synthesis in monolayer cultures of rat hepatocytes by induction of cholesterol 7 $\alpha$ -hydroxylase. *Biochem. J.* **262**, 341–348.
- Princen, H.M.G., Meijer, P., Wolthers, B.G., Vonk, R.J. and Kuipers, F. (1991) Cyclosporin A blocks bile acid synthesis in cultured hepatocytes by specific inhibition of chenodeoxycholic acid synthesis. *Biochem. J.* **275**, 501–505.
- Princen, H.M.G., Post, S.M. and Twisk, J. (1997) Regulation of bile acid biosynthesis. *Curr. Pharmaceut. Design* **3**, 59–84.

- Rao, Y.-P., Stravitz, R.T., Vlahcevic, Z.R., Gurley, E.C., Sando, J.J. and Hylemon, P.B. (1997) Activation of protein kinase Ca and S by bile acids: correlation with bile acid structure and diacylglycerol formation. *J. Lipid Res.* **38**, 2446–2454.
- Reiss, A.B., Martin, K.O., Javitt, N.B., Martin, D.W., Grossi, E.A. and Galloway, A.C. (1994) Sterol 27-hydroxylase: high levels of activity in vascular endothelium. *J. Lipid Res.* **35**, 1026–1030.
- Reiss, A.B., Martin, K.O., Rojer, D.E., Iyer, S., Grossi, E.A., Galloway, A.C. and Javitt, N.B. (1997) Sterol 27-hydroxylase: expression in human arterial endothelium. *J. Lipid Res.* **38**, 1254–1260.
- Rigotti, A., Trigatti, B.L., Penman, M., Rayburn, H., Herz, J. and Krieger, M. (1997) A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12610–12615.
- Rose, K.A., Stapleton, G., Dott, K., Kieny, M.P., Best, R., Schwarz, M., Russell, D.W., Bjorkhem, I., Seckl, J. and Lathe, R. (1997) Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7 $\alpha$ -hydroxydehydroepiandrosterone and 7 $\alpha$ -hydroxypregnenolone. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4925–4930.
- Rosen, H., Reshef, A., Maeda, N., Lippoldt, A., Shpizen, S., Triger, L., Eggertsen, G., Bjorkhem, I. and Leitersdorf, E. (1998) Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J. Biol. Chem.* **273**, 14805–14812.
- Rudel, L., Deckelman, C., Wilson, M., Scobey, M. and Anderson, R. (1994) Dietary cholesterol and downregulation of cholesterol 7 $\alpha$ -hydroxylase and cholesterol absorption in African green monkeys. *J. Clin. Invest.* **93**, 2463–2472.
- Rudling, M., Parini, P. and Angelin, B. (1997) Growth hormones and bile acid synthesis: key role for the activity of hepatic microsomal cholesterol 7 $\alpha$ -hydroxylase in the rat. *J. Clin. Invest.* **99**, 2239–2245.
- Russell, D.W. and Setchell, K.D.R. (1992) Bile acid biosynthesis. *Biochemistry* **31**, 4737–4749.
- Schwarz, M., Lund, E.G., Lathe, R., Bjorkhem, I. and Russell, D.W. (1997) Identification and characterization of a mouse oxysterol 7 $\alpha$ -hydroxylase cDNA. *J. Biol. Chem.* **272**, 23995–24001.
- Schwarz, M., Lund, E.G. and Russell, D.W. (1998a) Two 7 $\alpha$ -hydroxylase enzymes in bile acid biosynthesis. *Curr. Opin. Lipidol.* **9**, 113–118.
- Schwarz, M., Lund, E.G., Setchell, K.D.R., Kayden, H.J., Zerwekh, J.E., Bjorkhem, I., Hertz, J. and Russell, D.W. (1996) Disruption of cholesterol 7 $\alpha$ -hydroxylase gene in mice: II. Bile acid deficiency is overcome by induction of oxysterol 7 $\alpha$ -hydroxylase. *J. Biol. Chem.* **271**, 18024–18031.
- Schwarz, M., Russell, D.W., Dietschy, J.M. and Turley, S.D. (1998b) Marked reduction in bile acid synthesis in cholesterol 7 $\alpha$ -hydroxylase-deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia. *J. Lipid Res.* **39**, 1833–1843.
- Segev, H., Reshef, A., Clavey, V., Delbart, C., Routier, G. and Leitersdorf, E. (1995) Premature termination codon at the sterol 27-hydroxylase gene causes cerebrotendinous xanthomatosis in a French family. *Hum. Genet.* **95**, 238–240.
- Setchell, K.D., Bragetti, P., Zimmer-Nechemias, L., Daugherty, C., Pelli, M.A., Vaccaro, R., Gentili, G., Distrutti, E., Dozzini, G., Morelli, A., *et al.* (1992) Oral bile acid treatment and the patient with Zellweger syndrome. *Hepatology* **15**, 198–207.
- Setchell, K.D., Suchy, F.J., Welsh, M.B., Zimmer-Nechemias, L., Heubi, J. and Balistreri, W.F. (1988)  $\Delta^4$ -<sup>3</sup>-oxosteroid 5 $\beta$ -reductase deficiency described in identical twins with neonatal hepatitis: a new inborn error in bile acid synthesis. *J. Clin. Invest.* **82**, 2148–2157.

- Setchell, K.D.R., Schwarz, M., O'Connell, N.C., Lund, E.G., Davis, D.L., Lathe, R., Thompson, H.R., Weslie-Tyson, R., Sokol, R.J. and Russell, D.W. (1998) Identification of a new inborn error in bile acid synthesis: mutation of the oxysterol 7 $\alpha$ -hydroxylase gene causes severe neonatal liver disease. *J. Clin. Invest.* **102**, 1690–1703.
- Shefer, S., Nguyen, L., Salen, G., Batta, A.K., Brooker, D., Zaki, F.G., Rani, I. and Tint, G.S. (1990) Feedback regulation of bile acid synthesis in the rat: differing effects of taurocholate and taurooursocolate. *J. Clin. Invest.* **85**, 1191–1198.
- Shefer, S., Salen, G., Batta, A.K., Honda, A., Tint, G.S., Irons, M., Elias, E.R., Chen, T.C. and Holick, M.F. (1995) Markedly inhibited 7-dehydrocholesterol- $\Delta^7$ -reductase activity in liver microsomes from Smith-Lemli-Opitz homozygotes. *J. Clin. Invest.* **96**, 1779–1785.
- Shinki, T., Shimada, H., Wakino, S., Anazawa, H., Hayashi, M., Saruta, T., DeLuca, H.F. and Suda, T. (1997) Cloning and expression of rat 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase cDNA. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12920–12925.
- Shneider, B.L., Setchell, K.D., Whittington, P.F., Neilson, K.A. and Suchy, F.J. (1994)  $\Delta^{4-3}$ -oxosteroid 5 $\beta$ -reductase deficiency causing neonatal liver failure and hemochromatosis. *J. Pediatr.* **124**, 234–238.
- Shoda, J., Toll, A., Axelson, M., Pieper, F., Wikvall, K. and Sjoval, J. (1993) Formation of 7 $\alpha$ - and 7 $\beta$ -hydroxylated bile acid precursors from 27-hydroxycholesterol in human liver microsomes and mitochondria. *Hepatology* **17**, 395–403.
- Stahlberg, D., Reihner, E., Rudling, M., Berglund, L., Einarsson, K. and Angelin, B. (1994) Influence of bezafibrate on hepatic cholesterol metabolism in gallstone patients: reduced activity of cholesterol 7 $\alpha$ -hydroxylase. *Hepatology* **21**, 1025–1030.
- Stapleton, G., Steel, M., Richardson, M., Mason, J.O., Rose, K.A., Morris, R.G.M. and Lathe, R. (1995) A novel cytochrome P450 expressed primarily in brain. *J. Biol. Chem.* **270**, 29739–29745.
- Straka, M.S., Junker, L.H., Zaccaro, L., Zogg, D.L., Dueland, S., Everson, G.T. and Davis, R.A. (1990) Substrate stimulation of 7 $\alpha$ -hydroxylase, an enzyme located in the cholesterol-poor endoplasmic reticulum. *J. Biol. Chem.* **265**, 7145–7149.
- Stravitz, R.T., Hylemon, P.B., Heuman, D.M., Hagey, L.R., Schteingart, C.D., Ton-Nu, H.-T., Hoffman, A.F. and Vlahcevic, Z.R. (1993) Transcriptional regulation of cholesterol 7 $\alpha$ -hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. *J. Biol. Chem.* **268**, 13987–13993.
- Stravitz, R.T., Vlahcevic, Z.R., Gurley, E.G. and Hylemons, P.B. (1995) Repression of cholesterol 7 $\alpha$ -hydroxylase transcription by bile acids is mediated through protein kinase C in primary cultures of rat hepatocytes. *J. Lipid Res.* **36**, 1359–1368.
- Stravitz, R.T., Vlahcevic, Z.R., Russell, T.L., Heizer, M.L., Avadhani, N.G. and Hylemon, P.B. (1996) Regulation of sterol 27-hydroxylase and an alternative pathway of bile acid biosynthesis in primary cultures of rat hepatocytes. *J. Steroid Biochem. Molec. Biol.* **57**, 337–347.
- Stroup, D., Grestani, M. and Chiang, J.Y.L. (1997a) Identification of a bile acid response element in the cholesterol 7 $\alpha$ -hydroxylase gene (CYP7A). *Am. J. Physiol.* **273**, G508–G517.
- Stroup, D., Crestani, M. and Chiang, J.Y.L. (1997b) Orphan Receptors chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TFII) and Retinoid X Receptor (RXR) activate and bind the rat cholesterol 7 $\alpha$ -hydroxylase gene (CYP7A). *J. Biol. Chem.* **272**, 9833–9839.
- Suchy, F.J., Sippel, C.J. and Ananthanarayana, M. (1997) Bile acid transport across the hepatocyte canalicular membrane. *FASEB J.* **11**, 199–205.
- Thompson, J.F., Lira, M.E., Lloyd, D.B., Hayes, L.S., Williams, S. and Elsenboss, L. (1993) Cholesterol 7 $\alpha$ -hydroxylase promoter separated from cyclophilin pseudogene by Alu sequence. *Biochim. Biophys. Acta* **1168**, 239–242.

- Tint, G.S., Irons, M., Elias, E.R., Batta, A.K., Frieden, R., Chen, T.S. and Salen, G. (1994) Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330**, 107–113.
- Toll, A., Shoda, J., Axelson, M., Sjoval, J. and Wikvall, K. (1992) 7 $\alpha$ -Hydroxylation of 26-hydroxycholesterol, 3 $\beta$ -hydroxy-5-cholestenoic acid and 3 $\beta$ -hydroxy-5-cholenoic acid by cytochrome P-450 in pig liver microsomes. *FEBS Lett.* **296**, 73–76.
- Toll, A., Wikvall, K., Sudjana-Sugiaman, E., Kondo, K. and Bjorkman, I. (1994) 7 $\alpha$ -hydroxylation of 25-hydroxycholesterol in liver microsomes: evidence that the enzyme involved is different from cholesterol 7 $\alpha$ -hydroxylase. *Eur. J. Biochem.* **224**, 309–316.
- Trauner, M., Meier, P.J. and Boyer, J.L. (1998) Molecular pathogenesis of cholestasis. *N. Engl. J. Med.* **339**, 1217–1227.
- Turley, S.D., Schwarz, M., Spady, O.K. and Dietschy, J.M. (1998) Gender-related differences in bile acid and sterol metabolism in outbred CD-1 mice fed low- and high-cholesterol diets. *Hepatology* **28**, 1088–1094.
- Twisk, J., deWit, E.C.M. and Princen, H.M.G. (1995a) Suppression of sterol 27-hydroxylase mRNA and transcriptional activity by bile acids in cultured rat hepatocytes. *Biochem. J.* **305**, 505–511.
- Twisk, J., Hoekman, M.F.M., Lehmann, E.M., Meijer, P., Mager, W.H. and Princen, H.M.G. (1995b) Insulin suppresses bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7 $\alpha$ -hydroxylase and sterol 27-hydroxylase gene transcription. *Hepatology* **21**, 501–510.
- Tzung, K., Ishimura-Oka, K., Kihara, S., Oka, K. and Chan, L. (1994) Structure of the mouse cholesterol 7 $\alpha$ -hydroxylase gene. *Genomics* **21**, 244–247.
- Usui, E., Noshiro, M., Ohyama, Y. and Okuda, K. (1990a) Molecular cloning of cDNA for vitamin D<sub>3</sub> 25-hydroxylase from rat liver mitochondria. *FEBS Lett.* **262**, 135–138.
- Usui, E., Noshiro, M., Ohyama, Y. and Okuda, K. (1990b) Unique property of liver mitochondrial P450 to catalyze the two physiologically important reactions involved in both cholesterol catabolism and vitamin D activation. *FEBS Lett.* **274**, 175–177.
- Vega, G.L., von Bergmann, K., Grundy, S.M., Beltz, W., Jahn, C. and East, C. (1987) Increased catabolism of VLDL-apolipoprotein B and synthesis of bile acids in a case of hypobetalipoproteinemia. *Metabolism* **36**, 262–269.
- Vlahcevic, Z.R., Jairath, S.K., Heuman, D.M., Stravitz, R.T., Hylemon, P.B., Avadhani, N.G., and Pandak, W.M. (1996) Transcriptional regulation of hepatic sterol 27-hydroxylase by bile acids. *Am. J. Physiol.* **270**, G646–G652.
- Vlahcevic, Z.R., Stravitz, R.T., Heuman, D.M., Hylemon, P.B. and Pandak, W.M. (1997) Quantitative estimations of the contribution of different bile acid pathways to total bile acid synthesis in the rat. *Gastroenterology* **113**, 1949–1957.
- Wang, D.P. and Chiang, J.Y.L. (1994) Structure and nucleotide sequences of the human cholesterol 7 $\alpha$ -hydroxylase gene (CYP7). *Genomics* **20**, 320–323.
- Wang, D.P., Stroup, D., Marrapodi, M., Crestani, M., Galli, G. and Chiang, J.Y.L. (1996) Transcriptional regulation of the human cholesterol 7 $\alpha$ -hydroxylase gene (CYP7A) in HepG2 cells. *J. Lipid Res.* **37**, 1831–1841.
- Wang, J., Freeman, D.J., Grundy, S.M., Levine, D.M., Guerra, R. and Cohen, J.C. (1998) Linkage between cholesterol 7 $\alpha$ -hydroxylase and high plasma low-density lipoprotein cholesterol concentration. *J. Clin. Invest.* **101**, 1283–1291.
- Wang, S.-L., Du, E.Z., Martin, T.D. and Davis, R.A. (1997) Coordinate regulation of lipogenesis, the assembly and secretion of apolipoprotein B-containing lipoproteins by sterol response elements binding protein I. *J. Biol. Chem.* **272**, 19351–19358.

- Willy, P., Umesono, K., Ong, E., Evans, R., Heyman, R. and Mangelsdorf, D. (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Devel.* **9**, 1033–1045.
- Wuarin, J. and Schibler, U. (1990) Expression of the liver enriched transcription activator protein DBP follows a stringent circadian rhythm. *Cell* **63**, 1257A-1266A.
- Xu, G., Salen, G., Batta, A.K., Shefer, S., Nguyen, L.B., Niemann, W., Chen, T.S., Arora-Mirchandani, R., Ness, G.C. and Tint, G.S. (1992) Glycocholic acid and glycodeoxycholic acid but not glycoursocholic acid inhibit bile acid synthesis in the rabbit. *Gastroenterology* **102**, 1717–1723.
- Xu, G., Salen, G., Nguyen, L.B., Tint, G.S. and Shefer, S. (1996) Hepatic bile acid pool regulates cholesterol 7 $\alpha$ -hydroxylase but not sterol 27-hydroxylase in cholesterol-fed New Zealand white rabbits. *Hepatology* **24**, 309A.
- Xu, G., Salen, G., Shefer, S., Ness, G.C., Nguyen, L.B., Parker, T.S., Zhao, Z., Donnelly, T.M. and Tint, G.S. (1995) Unexpected inhibition of cholesterol 7 $\alpha$ -hydroxylase by cholesterol in New Zealand white and Watanabe heritable hyperlipidemic rabbits. *J. Clin. Invest.* **95**, 1497–1504.
- Xu, G., Salen, G., Shefer, S., Tint, G.S., Nguyen, L.B., Chen, T.S. and Greenblatt, D. (1999) Increasing dietary cholesterol induces different regulation of classic and alternative bile acid synthesis. *J. Clin. Invest.* **103**, 89–95.
- Xu, G., Salen, G., Shefer, S., Tint, G.S., Nguyen, L.B., Parker, T.T., Chen, T.S., Roberts, J., Kong, X. and Greenblatt, D. (1998) Regulation of classic and alternative bile acid synthesis in hyper-cholesterolemic rabbits: effects of cholesterol feeding and bile acid depletion. *J. Lipid Res.* **39**, 1608–1615.
- Zhang, J., Akwa, Y., Baulieu, E.E. and Sjovall, J. (1995a) 7 $\alpha$ -hydroxylation of 27-hydroxycholesterol in rat brain microsomes. *Compt. Rend. Acad. Sci.-Serie Iii, Sciences de la Vie* **318**, 345–349.
- Zhang, J., Larrson, O. and Sjovall, J. (1995b) 7 $\alpha$ -Hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol in human fibroblasts. *Biochim. Biophys. Acta* **1995**, 353–359.

## 5.

# CHOLESTEROL METABOLISM IN STEROIDOGENIC TISSUES

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The study of diseases of cholesterol metabolism (i.e., familial hypercholesteremia, cholesterol ester storage disease (Wolman's disease), Smith-Lemli-Opitz syndrome, Niemann-Pick type C disease) has provided valuable insight into mechanisms of cholesterol (lipoprotein) uptake, biosynthesis and storage, and intracellular trafficking. Molecular genetic studies of these diseases identified key genes and led to a greater understanding of how the proteins they encode govern cholesterol dynamics. The discovery of steroidogenic acute regulatory protein (StAR) through the careful and persistent pursuit of laboratory investigation and the recognition that mutations in the StAR gene cause the rare disease of steroidogenesis, congenital lipoid adrenal hyperplasia, solved a longstanding enigma in the regulation of steroidogenesis: the identity of the "labile" protein required for cholesterol movement from the outer membrane to the inner mitochondrial membrane. The current knowledge of cellular cholesterol dynamics is reviewed, emphasizing lessons learned from the study of human diseases affecting cholesterol metabolism, with particular emphasis on the rate limiting step of steroidogenesis, the delivery of cholesterol to the inner mitochondrial membrane, which is stimulated by StAR.

KEY WORDS: cholesterol metabolism, steroidogenic acute regulatory protein, Niemann-Pick type C, congenital lipoid adrenal hyperplasia, cholesterol ester storage disease, familial hypercholesteremia.

### INTRODUCTION

Cholesterol is an essential structural element of cellular membranes and is the precursor of all steroid hormones and bile acids. The study of inherited human defects in cholesterol metabolism has led to a greater understanding of the role of cholesterol in steroidogenesis and its importance to the homeostasis of all cells. This chapter summarizes the pathophysiology of several human genetic defects and the impact on our understanding of cholesterol transport into and within steroidogenic cells. Additionally, the current concepts regarding intracellular transport of cholesterol will be discussed with emphasis on the rate-limiting step in steroidogenesis, the movement of cholesterol from the outer to the inner mitochondrial membrane.

## CHOLESTEROL

Cells and plasma lipoproteins contain two forms of “cholesterol”, namely free cholesterol and cholesterol esters. Free cholesterol is the proximate precursor for steroidogenesis; it is nonuniformly distributed in cell membranes, concentrated primarily in the plasma membrane and to a lesser extent in intracellular organelle membranes (see reviews by Liscum and Underwood (1995) and Schroeder *et al.* (1996)). The cholesterol/phospholipid ratios of membranes vary over a 30-fold range: 0.03 in mitochondrial membranes; 0.08 in endoplasmic reticulum; 0.49 in lysosomal membranes; 0.4–0.76 in plasma membranes (Schroeder *et al.*, 1996). Moreover, even within the membrane bilayer, free cholesterol is asymmetrically distributed (Liscum and Underwood, 1995). This is highlighted in comparisons of the exofacial and cytofacial leaflets of the plasma membrane, where cholesterol levels may be enriched as much as 7-fold in the cytofacial leaflet (Schroeder *et al.*, 1996). More remarkable are estimates by Lange (1993) that indicate the entire plasma membrane pool of cholesterol cycles to the endoplasmic reticulum and back to the plasma membrane with a half-time of 40 min.

Cholesterol esters, in which cholesterol is esterified primarily to long-chain polyunsaturated fatty acids and to a lesser extent sulfate, make up the other predominant form of “cholesterol” (Fielding and Fielding, 1997). Cholesterol esters are found in cytoplasmic droplets and in the core of lipoprotein particles and account for 60–80% of the total cholesterol content of lipoproteins. Lipoprotein free cholesterol is localized to the particle protein-phospholipid coat. Cholesterol esters, which cannot replace free cholesterol in cell membranes nor be directly converted into steroid hormones, appear to be primarily depots of excess cellular sterol.

Much of our understanding of the intracellular movement and importance of these two forms of cholesterol has been derived from the elucidation of the etiology of inherited metabolic defects. However, before discussing these defects and the important lessons that they teach us about cellular cholesterol homeostasis, a general description of a typical steroidogenic cell is needed to provide a context for understanding how perturbations in cholesterol metabolism caused by inherited defects of key genes influence cell architecture and function. Additionally, this description highlights aspects of steroidogenic cell function that are not yet understood.

## STRUCTURE OF THE STEROIDOGENIC CELL

Steroidogenic cells exhibit many unique structural characteristics that enhance their ability to obtain the raw materials (i.e., cholesterol) and process these materials into a final product (i.e., steroid hormones). Steroidogenic cells are characterized by a plasma membrane containing numerous microvilli and clathrin-coated pits (Paavola *et al.*, 1985). Located on these microvilli are the cholesterol gathering LDL receptor family members (Nestler *et al.*,

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1992). Receptors for high density lipoproteins (HDL), members of the scavenger receptor type B class 1 (SR-B1), are also preferentially localized on the surface of steroidogenic cells and liver cells, where they selectively extract cholesterol esters from HDL, an important source of substrate for steroidogenesis and bile acid formation, respectively (Acton *et al.*, 1996). The plasma membrane is the single largest depot of free cholesterol (Warnock *et al.*, 1993) and is the site of receptors for the pituitary-derived trophic hormones (e.g., FSH, LH and ACTH) which play key roles in modulating the genes involved in cholesterol uptake, transport and utilization within steroidogenic cells (Richards, 1994). The plasma membrane is not a static structure and its cholesterol regularly cycles through the cell and back to the plasma membrane.

Steroidogenic cells are also characterized by abundant smooth endoplasmic reticulum (SER) (Enders, 1973). The SER takes on unique forms (whorls) within certain steroidogenic cell types. Steroidogenic cells can contain up to 10-fold more SER than rough ER, whereas nonsteroidogenic cells typically contain very little SER and significant amounts of rough ER. The disproportionate amounts of SER house the steroidogenic enzymes, 3 $\beta$ -hydroxysteroid dehydrogenase, CYP17, CYP21 and CYP19 (Hall, 1986). Moreover, the SER is the site where HMG-CoA reductase, the rate limiting enzyme in *de novo* cholesterol synthesis, as well as other enzymes participating in cholesterol production, reside (Reinhart *et al.*, 1987). Interestingly, in human granulosa cells the SER content increases concurrently with the dramatic increases in steroidogenic synthetic capacity during the luteinization process. The factors driving the formation of the extensive amounts of SER within these tissues are not understood. The expression of unique SER proteins and/or the SER-associated steroidogenic enzymes and HMG-CoA reductase, an integral membrane protein, may stabilize the lipid bilayer and promote the accumulation of the SER.

Steroidogenic cells also contain a large number of mitochondria which exhibit a unique morphology, being spherical in shape with tubulovesicular criste versus the typical elongated mitochondria with lamellar criste of non-steroidogenic cells (Belloni *et al.*, 1987; Enders, 1973). In luteinizing granulosa cells, which are acquiring the ability to produce large amounts of steroid hormone from cholesterol, the mitochondria change from the elongated forms with lamellar cristae to the spherical-tubular-vesicular form. It is not known what causes this unique mitochondrial transformation, nor is it understood how this configuration influences steroidogenesis. Normal mitochondrial function is paramount to normal steroidogenesis because the matrix side of the inner mitochondrial membrane is the location of the P450 side-chain cleavage enzyme (CYP11A, also known as P450<sub>scc</sub>) and its associated flavoprotein and iron sulfur electron transport system which converts cholesterol to pregnenolone (Hall, 1986). The reaction catalyzed by CYP11A represents the first committed step in steroidogenesis.

Electron microscopic analyses of mitochondrial membranes of steroidogenic cells suggest the presence of irregularly spaced contact sites between the inner and outer membranes (Cherradi *et al.*, 1997; Stocco and Clark, 1996). It has been postulated that these contact sites might be areas where cholesterol could move from the relatively cholesterol-rich outer membrane to the cholesterol-poor inner membrane. However, the existence of these contact sites (areas where the inner and outer membranes are fused) has recently been challenged and it has been proposed that they are an artifact of the fixation and staining

procedures. Using high voltage electron microscopy and three-dimensional reconstruction techniques (tomography) of unfixed and unstained frozen mitochondria, Mannella (1999) observed the presence of irregularly spaced bridges, likely of protein origin, linking the intact outer and inner membranes. These bridges may be sites of sterol transfer and aggregation of steroidogenic enzymes.

Cytoplasmic lipid droplets are found in most steroidogenic cells (Belloni *et al.*, 1987; Nussdorfer, 1986). These droplets are primarily composed of cholesterol esters as well as some triglycerides and phospholipids surrounded by perilipins, phosphoproteins that may anchor lipid droplets to enzymes or cytoskeletal structures (Londos *et al.*, 1999). Perilipins are phosphorylated by protein kinase A in response to elevations in cAMP (Londos *et al.*, 1995; Servetnick *et al.*, 1995). As much as 80% of the total cholesterol content of steroidogenic cells can be found in lipid droplets as cholesterol esters. The formation of the lipid droplets in human granulosa cells occurs simultaneously with the acquisition of enzymes necessary for production of progesterone, the major steroid product of the corpus luteum. The subcellular components involved in lipid droplet formation are still not well understood. One possible explanation for the occurrence of these lipid droplets is the unique ability of steroidogenic cells to continue to produce LDL-receptors and take up LDL in the face of high intracellular cholesterol levels (Golos and Strauss, 1988). Typically, high intracellular cholesterol levels cause the down-regulation of LDL-receptor formation as well as other genes involved in cholesterol synthesis, such as HMG-CoA reductase (Osborne, 1995). In steroid hormone-producing cells LDL-receptor down-regulation is only modestly dampened even though intracellular levels of cholesterol are sufficient to completely down-regulate the HMG-CoA reductase gene (Golos and Strauss, 1988). Moreover, reducing circulating cholesterol levels *in vivo* causes a concomitant decrease in ovarian and adrenal lipid droplets (Nestler *et al.*, 1992 and references therein).

The lipid droplets are an important source of stored cholesterol for steroidogenesis, as evidenced by the reduction in lipid droplet formation in cells cultured in the lipoprotein-deficient medium and the concomitant reduction in steroidogenesis (Nestler *et al.*, 1992). Conversely, if steroid synthesis is inhibited by administration of aminoglutethimide, a CYP11A (P450<sub>scc</sub>) inhibitor, lipid droplets increase as does cholesterol ester content within the cell (Tavani *et al.*, 1982).

A large body of evidence suggests a functional role for the cytoskeleton in the transport of cholesterol through the cytoplasmic space to the mitochondria (Hall and Almahbobi, 1997; Schweitzer and Evans, 1998). The association of the intermediate filament protein, vimentin, with the lipid droplets in steroidogenic cells prompted the hypothesis that these filaments are somehow involved in cholesterol transport (Almahbobi *et al.*, 1992b). Vimentin preparations derived from steroidogenic cells also contain a significant contamination with lipids. Furthermore, *in vitro* studies with vimentin demonstrated that the amino-terminal head domain of vimentin is the site of lipid interaction (Perides *et al.*, 1987). Interestingly, the stability of this interaction was greatest for cholesterol esters, the major constituent of lipid droplets, although diacylglycerol, cholesterol, oxysterols and vitamin D<sub>3</sub>, were also shown to associate with vimentin. Subsequent immunohistochemistry studies in adrenal cells indicated that intermediate filaments were also associated with mitochondria (Almahbobi *et al.*, 1992a). Additional studies using the vimentin-deficient human adrenal

tumor cell line, SW-13, indicated that these cells required vimentin for utilization of LDL-derived cholesterol and that vimentin was essential for the movement of cholesterol from lysosomes to the site of esterification (Sarria *et al.*, 1992). However, in mice homozygous for a vimentin null mutation, no obvious phenotypic defects were observed in lipid droplet formation nor has any change in steroid hormone biosynthesis or cholesterol metabolism been reported (Colucci-Guyon *et al.*, 1994). These vimentin deletion studies do not rule out the importance of intermediate filaments in steroidogenesis, but do point out that cells can compensate for the loss of vimentin. For example vimentin-negative steroidogenic cells could increase endogenous cholesterol synthesis which does not require intermediate filaments (i.e., vimentin) for movement within the intracellular space.

Cholesterol transport varies distinctly depending on whether the sterol is endogenously synthesized or is derived from an exogenous source (Liscum and Munn, 1999). Trafficking of endogenously synthesized cholesterol from the endoplasmic reticulum to the plasma membrane is a rapid, energy- and temperature-dependent process that has been shown to be unaffected by drugs that disrupt the cytoskeleton or inhibit lysosomal function (Liscum and Munn, 1999). Additionally, the movement of cholesterol from the endoplasmic reticulum to the plasma membrane does not depend on a functional return system (Jacobs *et al.*, 1997). Studies by Uittenbogaard (1998) indicate that a complex of 4 proteins (caveolin, cyclophilin A, cyclophilin 40 and heat shock protein 56) and cholesterol compose an intracellular intermediate hypothesized to be the transporter of cholesterol derived from the endoplasmic reticulum to the plasma membrane caveolae. Caveolae are deep plasma membrane invaginations coated with the cholesterol binding protein, caveolin. Cells with few lipoprotein receptors have increased numbers of caveolae and amounts of caveolin (Fielding and Fielding, 1997).

### DE NOVO CHOLESTEROL SYNTHESIS

The ability to synthesize cholesterol *de novo* from acetyl-CoA is a feature of every nucleated cell type, underscoring the importance of cholesterol to normal cellular function. In fact, mutations of genes involved in the cholesterol biosynthetic pathway are thought to occur relatively infrequently and result in severe congenital abnormalities and often death soon after birth (Schafer *et al.*, 1992; FitzPatrick *et al.*, 1998; Tozawa *et al.*, 1999; Moebius *et al.*, 2000).

The recent elucidation of the molecular etiology of Smith-Lemli-Opitz (SLO) syndrome (a.k.a. RSH syndrome), a disorder clinically characterized by facial dysmorphogenesis, mental retardation, and multiple congenital anomalies, including ambiguous external genitalia in affected males, demonstrated that the disorder occurred as a result of a block in cholesterol biosynthesis (Opitz, 1994; Tint *et al.*, 1994). Chasalow (1985) and McKeever (1990) first observed that steroid/cholesterol metabolism might be linked to SLO syndrome as evidenced by abnormal fetal adrenal production of androgens leading to reduced estriol production. Reduced fetal testicular androgen production accounts for the ambiguous genitalia in 46XY subjects. However, equine type estriols account for over half the estrogens excreted by women hosting an SLO fetus. These 1,3,5(10), 7-estratetraene compounds are formed by metabolism of 7-dehydrocholesterol by steroidogenic enzymes

demonstrating that CYP11A (P450<sub>scc</sub>), 3 $\beta$ -hydroxysterol dehydrogenase, CYP17 and CYP19 can all act on 7-dehydro metabolites (Shackleton *et al.*, 1999). More recently, Andersson (1999) reported adrenal insufficiency in SLO subjects. Tint (1994) determined that SLO patients had abnormally low plasma cholesterol levels and excessive levels of 7-dehydrocholesterol, the immediate precursor to cholesterol. Subsequent studies indicated that tissue (adrenal, kidney, cerebral cortex and abdominal wall) and serum cholesterol levels were severely decreased ( $\sim$ 10-fold) in SLO patients (Tint *et al.*, 1995). Conversely, tissue and serum 7-dehydrocholesterol levels were markedly elevated in SLO patients, while no 7-dehydrocholesterol could be detected in age-matched control patient samples. These studies pointed to either a direct defect in 7-dehydrocholesterol reductase, an enzyme which reduces the  $\Delta^7$ -double bond of 7-dehydrocholesterol to produce cholesterol, or defects in genes that encode proteins essential for the expression and function of 7-dehydrocholesterol reductase.

Nearly thirty five years after the initial descriptions of Smith-Lemli-Opitz syndrome, the cDNA for 7-dehydrocholesterol reductase was cloned and shown to contain an open reading frame of 1425bp coding for a protein of 475 amino acids (Wassif *et al.*, 1998; Waterham *et al.*, 1998). Chromosomal mapping localized the gene to chromosome 11 (11q12–13). Furthermore, it was demonstrated that this fairly frequent (1 in 20,000) autosomal recessive developmental disorder was the result of distinct mutations that included a 134bp insertion and different point mutations in unrelated patients (Wassif *et al.*, 1998; Waterham *et al.*, 1998). These studies clearly indicate that *de novo* cholesterol synthesis plays a critical role in organ development, as well as in steroidogenesis.

#### DE NOVO CHOLESTEROL SYNTHESIS VERSUS UPTAKE OF LIPOPROTEIN-CARRIED CHOLESTEROL

The first challenge to steroid producing cells is obtaining the precursor cholesterol. While it is well established that human steroidogenic cells have the capability to produce cholesterol *de novo*, this method of obtaining cholesterol is thought to play a minor role in normally functioning organs. This premise is supported by several observations. Normal cells/tissues typically express low levels of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, as well as other enzymes essential for cholesterol synthesis. Secondly, human subjects given HMG-CoA reductase inhibitors fail to exhibit significant changes in adrenal or testicular steroid output (Laue *et al.*, 1987; Jay *et al.*, 1991). Thirdly, *in vitro* studies with fetal adrenal cells examining the rates of *de novo* cholesterol synthesis simultaneously with lipoprotein uptake suggest that *de novo* synthesis contributes less than 30% of the cholesterol used for steroidogenesis (Carr and Simpson, 1981). Lastly, diseases which affect the synthesis of lipoproteins or their uptake (i.e., the alternative cholesterol source) are known to impact steroidogenesis.

#### CHOLESTEROL TRANSPORT INTO STEROIDOGENIC CELLS

The mechanism of cholesterol uptake by LDL-receptor-mediated endocytosis is well established and has been extensively reviewed (Goldstein *et al.*, 1995). The itinerary of this

pathway was unraveled from the study of familial hypercholesteremia, a disease characterized clinically by marked elevations in serum levels of LDL and deposition of LDL-derived cholesterol in tendons, skin and arteries. The disease generally (in >95% of affected subjects) results from a defect in the LDL receptor, while the remaining 2–5% of these cases result from a defect in apolipoprotein B (apo B). Recently, evidence for a third genetic locus causing familial hypercholesteremia was identified in a kindred without LDL-receptor and apo B mutations (Haddad *et al.*, 1999).

Hypercholesteremia caused by LDL-receptor defects is inherited as an autosomal dominant trait with a gene dosage effect, such that homozygotes are more severely affected than heterozygotes. The LDL-receptor gene on the short arm of chromosome 19 has 18 exons that span 45 kb and produces a mature single-chain glycoprotein of 839 amino acids. Five general classes of mutations that effect LDL-receptor function/synthesis have been identified (Goldstein *et al.*, 1995): (1) these mutations result in null mutants that fail to produce proteins; (2) mutant proteins in which intracellular transport of precursor proteins is blocked; (3) receptors unable to bind LDL; (4) receptors that bind LDL but fail to initiate the clustering into clathrin-coated pits and thus fail to enter the cell; and (5) receptors which bind and internalize LDL but fail to release LDL in the endosome.

Hypercholesteremia can also result from a mutation in the apolipoprotein B (apo B) gene, the gene encoding the major apolipoprotein constituent of serum low-density lipoproteins. Full-length apo B protein consists of 4536 amino acids and is referred to as apo B-100. The apo B gene is located on the short arm of human chromosome 2 (2p23–2p24) (Deeb *et al.*, 1986). Hypercholesteremia resulting from a defective apo B gene is called familial defective apoB-100 and has been shown in the majority of cases to result from a single mutation in which an arginine is replaced by a glutamine at the 3500 amino acid residue of the protein (Innerarity *et al.*, 1990). This disease is specific to western Europeans and their descendents (Myant, 1993). The mutation prevents apo B from binding to the LDL-receptor, thus leading to increases in circulating serum cholesterol levels. Several other apo B mutations that lead to hypercholesteremia are known, but are extremely rare (Pullinger *et al.*, 1995; Ludwig *et al.*, 1997).

In patients with familial homozygous hypercholesterolemia, steroidogenesis by the adrenal glands and gonads is modestly impaired (Illingworth *et al.*, 1983; Boizel *et al.*, 1986). This is particularly evident following trophic hormonal stimulation of the glands, such as that observed following the periovulatory LH surge, or a stressor which prompts the release of ACTH. However, these patients have no clinically relevant reproductive or adrenal dysfunction presumably because of the enhanced *de novo* synthesis of steroids and/or uptake of cholesterol via the HDL receptors. Subsequent studies using inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, such as pravastatin, cholestyramine, lovastatin (mevinolin) in patients with familial hypercholesteremia indicated that gonadal and adrenal function was unaffected (Farnsworth *et al.*, 1987; Laue *et al.*, 1987; Jay *et al.*, 1991). These data suggest that enhancement of *de novo* cholesterol synthesis is not the only way cells can make up for a loss in LDL receptor function and in instances where endogenous cholesterol synthesis is blocked, alternative mechanisms for increasing cholesterol uptake are invoked by the scavenger receptors. Selective cholesterol ester uptake, a phenomena where cholesterol esters from HDL and LDL are directly taken up by

the cell in the absence of lipoprotein particle internalization, could sustain steroid production in these individuals (*vide infra*).

The importance of LDL-derived cholesterol to steroidogenesis is highlighted by the observations in patients who have familial hypobetalipoproteinemia, a disease characterized by the virtual absence of any circulating very low density lipoproteins and LDL. In these patients, maximal steroid output in response to trophic hormones is far below that observed in controls (Illingworth *et al.*, 1982b). Furthermore, patients with hypobetalipoproteinemia have markedly lower progesterone levels during the menstrual cycle and pregnancies (Illingworth *et al.*, 1982a; Parker *et al.*, 1986). Approximately 35 different mutations of the apo B gene that specify null alleles or truncated protein have been described (Farese *et al.*, 1992; Schonfeld, 1995). However, and even more remarkably, in a large group of individuals and kindred with low serum cholesterol levels only 0.5% resulted from known truncation mutants of apo B (Wu *et al.*, 1999), suggesting an even greater number of mutations will be identified. Alternatively, this finding could suggest the presence of additional dysfunctional regulatory mechanisms involved in either apo B production and/or function.

While the role of the HDL receptors in steroidogenesis was not disclosed by the clinical abnormalities resulting from a mutation in apolipoprotein A-I, the major apolipoprotein found in HDL, targeted deletion of the gene indicated that HDL particles play an important role in the provision of substrate for steroidogenesis in mice (Plump *et al.*, 1996). However, it is important to note that in mice, HDL serves as the major source of cholesterol for steroid synthesis, whereas LDL is a minor source, in contrast to the human (Gwynne and Strauss, 1982). The cloning of the scavenger receptor BI (SR-BI) clarified the mechanism of HDL sterol uptake by steroidogenic cells (Acton *et al.*, 1994). This receptor has a high affinity for HDL and mediates the selective uptake of cholesterol esters from HDL (Acton *et al.*, 1996). Targeted deletion of the SR-BI gene demonstrated that female mice were infertile (Trigatti *et al.*, 1999). These mice exhibited reduced lipid levels in the corpora lutea and the adrenal cortex as assessed by oil red O staining, suggesting a reduction in cholesterol ester storage. The decline in fertility, however, could not be attributed to reduced steroid output as endocrine profiles were normal. Moreover, male mice exhibited normal fertility and normal corticosteroid production (Rigotti *et al.*, 1997). The SR-BI human homolog called CLA-1 like SR-BI is highly expressed in the adrenal gland, liver and testis (Murao *et al.*, 1997). Moreover, it was confirmed that CLA-1 was functionally related to SR-BI because it bound HDL with high affinity and mediated the selective transport of cholesterol esters into cells (Murao *et al.*, 1997). The mechanisms by which SR-BI mediates the uptake of cholesterol ester from HDL are not clearly understood, but a series of reports reviewed by Fidge (1999) favor a selective non-endocytotic pathway for delivery of HDL-associated cholesterol esters. This model was supported by the lack of uptake of radiolabeled apolipoprotein A-I by cells transfected with CLA-1, while radiolabeled cholesterol esters were found to accumulate. These cholesterol esters presumably are then hydrolyzed by extralysosomal lipases and are then able to enter the intracellular cholesterol regulatory and storage pools (Sparrow and Pittman, 1990). In addition to the selective uptake of cholesterol esters from HDL, selective uptake of cholesterol esters from LDL by gonadal tissue was also recently demonstrated (Azhar *et al.*, 1999; Stangl *et al.*, 1999). The

quantitative importance of selective cholesterol uptake from either HDL or LDL in human steroidogenic tissues remains to be evaluated.

### CHOLESTEROL ESTER FORMATION

The esterification of cellular cholesterol with fatty acids is catalyzed by the microsomal enzyme acyl-CoA:cholesterol acyltransferase (ACAT). There are two distinct ACATs encoded by different genes. ACAT1 is responsible for cholesterol esterification in steroidogenic glands (Farese, 1998; Rudel *et al.*, 2001). As ACAT-mediated synthesis of sterol esters progresses, the esters accumulate within the endoplasmic reticulum membranes and bud off as lipid droplets. The importance of ACAT in cholesterol ester storage has been revealed by targeted deletion of the ACAT gene in mice which causes a marked reduction in adrenal cholesterol ester stores without impairing basal or ACTH-stimulated glucocorticoid synthesis (Meiner *et al.*, 1996).

### CHOLESTEROL ESTER HYDROLYSIS: LYSOSOMAL

After uptake of the LDL-receptor-clathrin coated pit complex, the clathrin coat is removed and the LDL-endosomes fuse to form multivesicular bodies which in turn fuse with acid hydrolase-containing primary lysosomal vesicles derived from the trans-Golgi network (reviewed by Assmann and Seedorf (1995) and Goldstein *et al.* (1995)). The acidification of the vesicle interna and hydrolysis of the apolipoproteins, cholesterol esters and other lipid modes frees the LDL-receptor so it can recycle back to the cell surface (Goldstein *et al.*, 1995). Some of the free cholesterol is redirected to the plasma membrane, while the rest enters the endoplasmic reticulum where it affects the transcriptional regulation of a variety of genes involved in cholesterol metabolism (e.g., LDL-receptor, HMG-CoA reductase and synthetase, squalene synthetase, etc.) and/or the cellular activity of the microsomal-bound ACAT, which leads to the formation of cholesterol esters, and in steroidogenic tissues the formation of lipid droplets.

The lysosomal degradation of cholesterol esters is catalyzed by acid lipase. Two diseases, Wolman disease and cholesterol ester storage disease (CESD), are characterized by a deficiency in the activity of the lysosomal acid lipase (Assmann and Seedorf, 1995). Wolman disease and cholesterol ester storage disease are autosomal recessive disorders; the key difference between these two related abnormalities is the clinical course. Wolman disease generally occurs in infancy and is nearly always fatal (Assmann and Seedorf, 1995). The cellular manifestation is characterized by a marked accumulation of both triglycerides and cholesterol esters within the lysosomes as would be predicted by the virtual absence of acid lipase. The inability to release free cholesterol causes the up-regulation of genes involved in cholesterol uptake (i.e., LDL receptor) and *de novo* cholesterol synthesis, which in turn further increases the cholesterol buildup in lysosomes, leading to a vicious cycle. The accumulated LDL and cholesterol can undergo oxidation, further damaging affected cells (Fitoussi *et al.*, 1994). In CESD, lysosomal acid lipase has some residual activity which frees enough cholesterol to modulate the negative feedback systems to partially down-regulate the LDL-receptor formation, thus preventing the vicious cycle observed in Wolman disease.

Human lysosomal acid lipase cDNA was cloned in 1991 (Anderson and Sando, 1991). The gene was localized by *in situ* hybridization to chromosome 10q23.2-q23.3 (Anderson *et al.*, 1994; Aslanidis *et al.*, 1994). It spans 38.8kb consisting of 10 exons that encode a 378 amino acid protein. The 5' flanking promoter region of the gene is GC rich, a characteristic of housekeeping genes.

Wolman disease almost always results from a homozygous mutation that leads to an inactive protein or a truncated protein missing the carboxy-terminus, which contains the catalytic domain (Pagani *et al.*, 1998; Lohse *et al.*, 1999). Because Wolman patients with heterozygous mutations are rare (Anderson *et al.*, 1994; Lohse *et al.*, 1999), the disease is thought to result from consanguinity from private mutations (Lohse *et al.*, 1999). In contrast, a single genetic defect, a splice junction mutation accounts for the majority (70%) of the CESD patients (Lohse *et al.*, 1999 and references therein). This mutation leads to the skipping of exon 8 and the loss of 24 amino acids from the mature protein. The mutation allows ~3% of normal splicing to occur ensuring the maintenance of residual lysosomal acid lipase activity necessary for the survival of the affected individual.

A mouse model for CESD and Wolman's has been produced by targeted deletion of lysosomal acid lipase (Du *et al.*, 1998). The homozygous null mutants had massive accumulations of cholesterol esters and triglycerides in the liver, thus presenting a histologically consistent manifestation of the human Wolman disease. However, these mice exhibited normal development into adulthood, unlike development observed in the human disease. Both male and female null mutant mice were fertile (Du *et al.*, 1998). It is plausible that steroidogenesis and fertility in mice are unaffected because HDL derived cholesterol, which bypasses the lysosomal pathway, acts as the precursor for steroidogenesis in this species. The impact of CESD on steroidogenesis, however, is thought to be minimized in humans because of the additional pathways for obtaining cholesterol: (1) *de novo* cholesterol synthesis; (2) selective uptake of cholesterol esters from LDL and HDL which bypass the lysosomal pathway; and (3) hydrolysis of stored cytoplasmic cholesterol esters by other cholesterol ester hydrolyase.

#### CHOLESTEROL ESTER HYDROLYSIS: EXTRALYSOSOMAL

Neutral cholesterol ester hydrolase (NCEH)(a.k.a. hormone sensitive lipase) is essential for the hydrolysis of cholesterol esters found in lipid droplets. Because lipid droplets are a source of cholesterol in steroidogenic tissues, NCEH is tightly regulated within these tissues by trophic hormones (LH, FSH, hCG)(Trzeciak *et al.*, 1984; Kraemer *et al.*, 1993; Aten *et al.*, 1995). NCEH is activated by protein kinase A dependent phosphorylation of serine residues on the protein (Nestler *et al.*, 1992). Targeted deletion of the NCEH gene does not result in abnormalities in female reproduction but does produce male infertility due to defects in the seminiferous epithelium and spermatogenesis (Chung *et al.*, 2001). The absence of accumulated sterol esters in steroidogenic cells in this model suggests the existence of alternative genes encoding sterol esterase activity.



## CHOLESTEROL MOVEMENT AFTER LYSOSOMAL HYDROLYSIS

After fusion of the primary lysosome with the LDL-cholesterol ester containing multivesicular organelles and the hydrolysis of the LDL components, the free cholesterol must get to the plasma membrane and the endoplasmic reticulum where it is incorporated into the regulatory sterol pool that ensures the induction of homeostatic responses. Much as the study of familial hypercholesteremia led to the isolation of the LDL receptor and our understanding of regulation of serum cholesterol levels, the study of the inherited Niemann-Pick type C (NPC) disease has contributed to our understanding of the metabolism of lysosomal free cholesterol (Neufeld *et al.*, 1999).

NPC disease, an autosomal recessive neurovisceral cellular lipidosis, is characterized by the inappropriate movement and partitioning of LDL-derived cholesterol through cellular organelles (Shamburek *et al.*, 1997; Xie *et al.*, 1999). The disease is associated with a build up of free cholesterol within the lysosomes and the Golgi complex (Pentchev *et al.*, 1997). Cytochemical and biochemical studies have shown that the transport of endocytosed LDL cholesterol out of the lysosomal compartment is affected in NPC disease. The NPC defect results in a reduced rate of transport of free cholesterol from the lysosomes to the plasma membrane (Neufeld *et al.*, 1999). Cytochemical evidence indicates that the NPC defect also affects cholesterol transport through the Golgi complex. HDL-derived cholesterol and the transport of endogenously synthesized cholesterol is unaffected in NPC cells (Shamburek *et al.*, 1997). Immunocytochemical studies demonstrated that the product of the NPC1 gene, which is mutated in the majority of cases of NPC disease, is localized in a subset of late endosomal vesicles containing lysosomal associated membrane protein-2 (a marker of late endosomal and lysosomal vesicles) and no mannose 6-phosphate receptors (another late endosomal marker). The NPC1 vesicles also contain rab7 and rab9, proteins involved in vesicular trafficking (Higgins *et al.*, 1999), and are distinct from the vesicles containing endocytosed cholesterol (Neufeld *et al.*, 1999). In NPC1 mutant cells or cells treated with hydrophobic amines or progesterone, which both induce a cell phenotype similar to that observed in NPC1 defective cells, NPC1 was immunolocalized in LAMP-2-positive cholesterol-laden vesicles. These findings and recent unpublished data examining trafficking of NPC1-green fluorescent protein fusion proteins indicate that the NPC1-containing vesicles unload lysosomal cargo through transient interaction with lysosomes. Mutant NPC1 proteins become trapped in lysosomes implicating NPC1 function in the trafficking of the NPC1 vesicles. Hydrophobic amines and progesterone also trap NPC1 in cholesterol-laden lysosomes.

The NPC1 gene located near the centromere of chromosome 18 (18q11) was identified by positional and complementation cloning (Carstea *et al.*, 1997). Complementation studies also indicated the existence of a second mutant gene, NPC2, which produces the NPC phenotype (Vanier *et al.*, 1996). The NPC2 gene was recently identified as the HE1 gene, that encodes a cholesterol binding protein highly expressed in the epididymis (Naureckiene *et al.*, 2000). The NPC1 gene has 25 exons and spans 47kb. To date 36 single nucleotide polymorphisms have been identified in unaffected individuals (Morris *et al.*, 1999). The 1278 amino acid gene product is an integral membrane glycoprotein as evidenced by the presence of an N-terminal signal sequence and 13 transmembrane domains. The carboxy terminus contains a lysosomal targeting motif (LLNF). The amino terminus of NPC1 is

highly conserved across species and has been named the NPC1 domain. The NPC1 domain is believed to be important for the unloading of lysosomal cargo and also contains a leucine zipper motif which is typically involved in protein-protein interactions. One of the more interesting characteristics of the NPC1 protein, however, is the sequence similarity to the sterol-sensing domains found in 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA)-reductase, SCAP the Sterol regulatory element-binding protein (SREBP)-Cleavage Activating Protein and Patched the interacting partner of Hedgehog (Loftus *et al.*, 1997). Structure-function analyses of the NPC1 protein indicate that mutations in the conserved cysteine residues of the NPC1 domain, the NPC1 domain leucine zipper motif, or in the sterol-sensing domain inactivate the glycoprotein (Watari *et al.*, 1999b). Furthermore, deletion of the lysosomal-targeting sequence at the C-terminus prevents the protein from reaching the lysosomes and prevents the mobilization of free cholesterol from these organelles (Watari *et al.*, 1999a).

The effects of the NPC phenotype on steroidogenesis have received little attention. It was determined that testosterone production is reduced in mice homozygous for mutations in the homologous NPC1 gene (Roff *et al.*, 1993). The Leydig cell defect was associated with marked engorgement of the interstitium with residual bodies that compress the cell cytoplasm and significantly reduce the capacity of these cells to generate testosterone from hydroxysterols and pregnenolone, reflecting diminished P450<sub>scc</sub>, P450<sub>c17</sub>, and 3 $\beta$ -hydroxy steroid dehydrogenase activities. A series of experiments by Watari *et al.* (2000) examined the role of NPC1 in LDL-stimulated progesterone synthesis by human granulosa-lutein cells. The hydrophobic amine, U18666A, was used to elicit the NPC-phenotype in the granulosa-lutein cells, as assessed by the pronounced increase in filipin staining (indicator of free cholesterol) in LAMP-2/NPC1 positive granules. LDL-stimulated progesterone synthesis was blocked by U18666A as was 8-Br-cAMP stimulated progesterone synthesis that was LDL-dependent. These data demonstrate that LDL-derived cholesterol supporting progesterone synthesis in granulosa-lutein cells passes through a U18666A-sensitive compartment; this compartment interacts with NPC1 and this pathway can be functionally separated from other intracellular pathways that contribute cholesterol substrate for steroidogenesis.

The role of NPC1 vesicles in the movement of cholesterol to the Golgi apparatus endoplasmic reticulum and plasma membranes is a subject of current investigation. The elucidation of these processes will shed much needed light on intracellular sterol trafficking.

Current thinking is focused on a model in which NPC1 acts as sterol "permease" in concert with the HE1 gene product which may function as a "sterol sink" or cofactor for the NPC1 permease (Davies *et al.*, 2000).

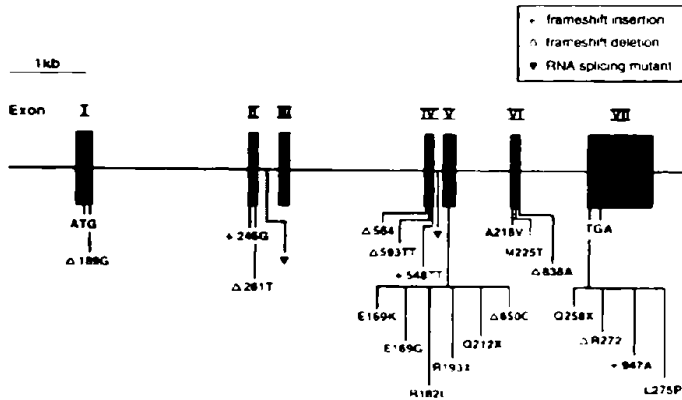
#### CHOLESTEROL TRANSPORT TO THE INNER MITOCHONDRIAL MEMBRANE

To initiate the final steps in the metabolism of cholesterol into steroid hormones, cholesterol must transverse the aqueous space between the cholesterol-rich outer mitochondrial membrane and cholesterol-poor inner membrane to reach the P450 side chain cleavage enzyme. In turn, P450<sub>scc</sub> and its associated electron transport proteins, which

reside on the matrix side of the inner mitochondrial membrane, convert cholesterol to pregnenolone, the first step in the biosynthesis of steroids and generally thought to be the rate-limiting step in steroidogenesis. However, early studies by Ferguson (1962, 1963) demonstrated that inhibitors of protein synthesis could block hormonal induction of steroidogenesis and subsequent studies in the late 1960s-1980s by many laboratories demonstrated that this block in steroidogenesis occurred prior to the formation of pregnenolone (see references within Strauss *et al.*, 1999). Privalle (1983) and Ohno (1983) demonstrated that the protein synthesis inhibitors effectively blocked the movement of cholesterol from the outer to inner mitochondrial membrane. In further studies, Orme-Johnston and colleagues identified a group of mitochondrial 30kDa phosphoproteins induced in rodent adrenal cells by ACTH and rodent gonadal cells by LH whose induction could be blocked by protein synthesis inhibitors and whose presence was directly correlated with steroidogenesis (see Strauss *et al.*, 1999). Identical proteins were found in dibutyryl-cAMP-stimulated MA-10 mouse Leydig cells by Stocco and colleagues (see reviews of Stocco, 1999 and Strauss *et al.*, 1999). The 30kDa proteins were shown to be derived from a 37kDa precursor synthesized in the cytoplasm and then imported into the mitochondria and processed to the 30kDa form. Subsequent purification of the 30kDa protein from MA-10 cells and amino acid sequence analysis allowed the cloning of the cDNA and the identification of a novel protein in the mouse and human (Clark *et al.*, 1994; Sugawara *et al.*, 1995a) referred to as steroidogenic acute regulatory protein (StAR). Again, as with the LDL receptor and NPC1, molecular genetics firmly established the role of this protein in intracellular cholesterol trafficking, when it was discovered that congenital lipid adrenal hyperplasia results from mutations in the *StAR* gene (Lin *et al.*, 1995).

Congenital lipid adrenal hyperplasia is the most severe form of congenital adrenal hyperplasia and is characterized by the marked reduction in biosynthesis of all adrenal and gonadal steroid hormones. The clinical phenotype of this disease includes the onset of profound adrenocortical insufficiency shortly after birth, hyperpigmentation reflecting increased production of pro-opiomelanocortin, elevated plasma renin activity as a consequence of reduced aldosterone synthesis, and male pseudohermaphroditism resulting from deficient fetal testicular testosterone synthesis (Hauffa *et al.*, 1985; Bose *et al.*, 1996; Miller and Strauss, 1999). The affected offspring are the products of uneventful pregnancies, delivered at term. Early in the disease the steroidogenic cells of the enlarged adrenal cortices in affected individuals are engorged with lipid droplets containing cholesterol esters, giving rise to the condition's name. Administration of ACTH or hCG to subjects with congenital lipid adrenal hyperplasia does not elicit the normal acute increase in serum levels of adrenal or gonadal steroid hormones.

Congenital lipid adrenal hyperplasia is a rare disease, except in Japan and Korea where it accounts for 5% or more of all cases of congenital adrenal hyperplasia (Nakae *et al.*, 1997; Yoo and Kim, 1998). Known to be inherited in an autosomal recessive pattern, congenital lipid adrenal hyperplasia was initially thought to be caused by mutations in the CYP11A (P450<sub>scc</sub>) gene. This supposition was further supported by observations in a rabbit model of human congenital lipid adrenal hyperplasia where a mutation of the CYP11A gene was detected and caused a loss of function of the CYP11A gene product (P450<sub>scc</sub>) (Pang *et al.*, 1992; Yang *et al.*, 1993; Iwamoto *et al.*, 1994). However, mitochondria isolated from

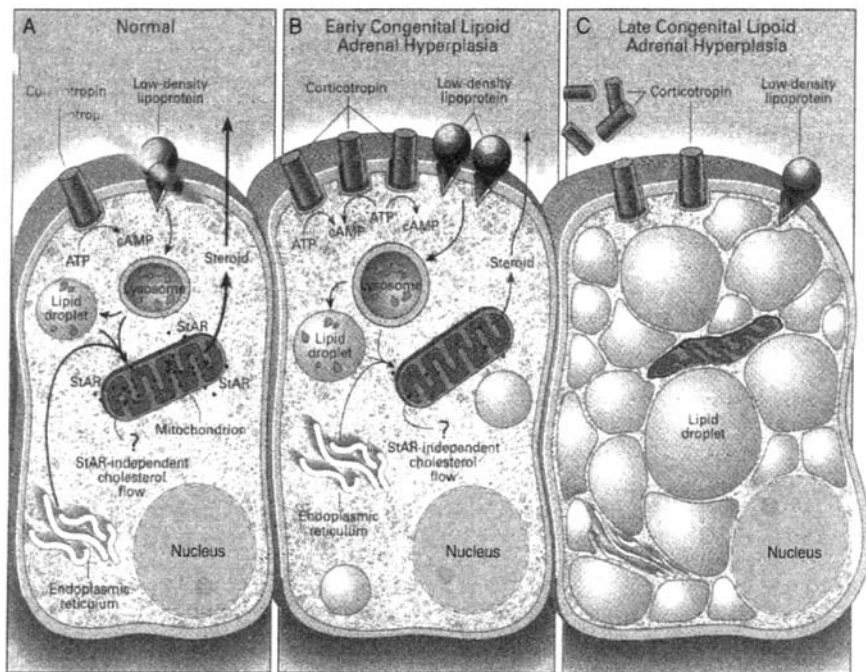


**Figure 5.1** Structure of the human StAR gene and mutations discovered in subjects with congenital lipid adrenal hyperplasia.

human adrenal tissue from affected individuals converted radiolabeled  $20\alpha$ -hydroxylcholesterol into pregnenolone, whereas radiolabeled cholesterol was not efficiently metabolized into pregnenolone, indicating the presence of a functional P450scc (Degenhart *et al.*, 1972). Later analysis of the CYP11A gene revealed that it is normal in individuals with congenital lipid adrenal hyperplasia and that the P450scc is present in testicular homogenates (Lin *et al.*, 1991; Saenger *et al.*, 1993; Sakai *et al.*, 1994).

The 8kb StAR gene was mapped to a single locus on human chromosome 8p11.2 and consisting of seven exons that code for a 284 (murine)/285 (human) amino acid protein (Clark *et al.*, 1994; Sugawara *et al.*, 1995a, b). The molecular genetic analysis of the StAR gene in more than 60 unrelated patients confirmed the presence of mutations in the StAR gene in the majority of these subjects (Lin *et al.*, 1995; Tee *et al.*, 1995; Bose *et al.*, 1996; Fujieda *et al.*, 1997; Nakae *et al.*, 1997; Okuyama *et al.*, 1997). Furthermore, analysis of DNA from the parents of several patients confirmed that this disease is inherited in an autosomal recessive pattern. The few patients diagnosed with congenital lipid adrenal hyperplasia that lacked mutations in the StAR gene may harbor mutations in regions of the gene that were not analyzed (i.e., the promoter). Alternatively, congenital lipid adrenal hyperplasia in those cases could result from mutations in other genes required for StAR function.

Mutations identified in the StAR gene include frame shifts caused by deletions/insertions, splicing errors and nonsense and missense mutations. All of these mutations lead to the absence of StAR protein or the production of functionally inactive StAR protein (see Figure 5.1). The molecular genetic studies of congenital lipid adrenal hyperplasia provided substantial insights into the structure/ function relationships of the StAR protein, indicating that the C-terminus of the protein is essential for its biological activity. Several nonsense mutations were shown to result in C-terminus truncations of StAR. One of these mutations, Q258X, accounts for 80% of the known mutant alleles in the affected Japanese population and results in the deletion of the final 28 amino acids of the StAR protein (Bose *et al.*, 1996; Nakae *et al.*, 1997). All of the known point mutations that result in amino acid substitutions occur in exons 5–7 of the gene, the exons that encode the C-terminus. Interestingly, even though StAR is taken into the mitochondrial inner membrane space and processed to its



**Figure 5.2 Model of the pathophysiology of congenital lipid adrenal hyperplasia.** (A) Normal adrenal cell. (B) Adrenal cell deficient in StAR at early stage of disease. The efficient flow of cholesterol to the inner mitochondrial membranes is impaired, leaving only a modest StAR-independent steroidogenesis. Consequently, corticotrophin secretion is increased, resulting in adrenal cortical hyperplasia and increased uptake of LDL cholesterol, with accumulation of internalized cholesterol in lipid droplets due to inefficient movement of the substrate to P450scc (CYP11A). (C) Late stage of disease, with massive accumulation of cholesterol in cytoplasmic lipid droplets, leading to organelle compression and auto-oxidation of cholesterol with subsequent peroxidative damage of proteins and organelles and a severe impairment of steroidogenesis. Reprinted with permission from Bose *et al.*, N. Engl. J. Med., 335, 1870–1878, 1996, Copyright The Massachusetts Medical Society.

mature form, all of these point mutations result in an inactive StAR protein as tested in a biological assay of steroidogenesis enhancement in COS-1 cells co-transfected with the cholesterol side-chain cleavage system. It is postulated that these mutant proteins are inactive due to folding errors caused by the loss of salt bridges that stabilize tertiary structure (Bose *et al.*, 1996, 1998). These findings indicate that the C-terminus of StAR is essential for steroidogenic activity and the entry of StAR and processing to the mature 30kDa form are not required for stimulation of pregnenolone synthesis.

The metabolic defect in congenital lipid adrenal hyperplasia appears to be progressive, with adrenal and gonadal steroidogenesis becoming increasingly impaired with time after birth. Bose *et al.* proposed a model (see Figure 5.2) to explain the pathophysiology of congenital lipid adrenal hyperplasia. This model reflects the existence of some StAR-

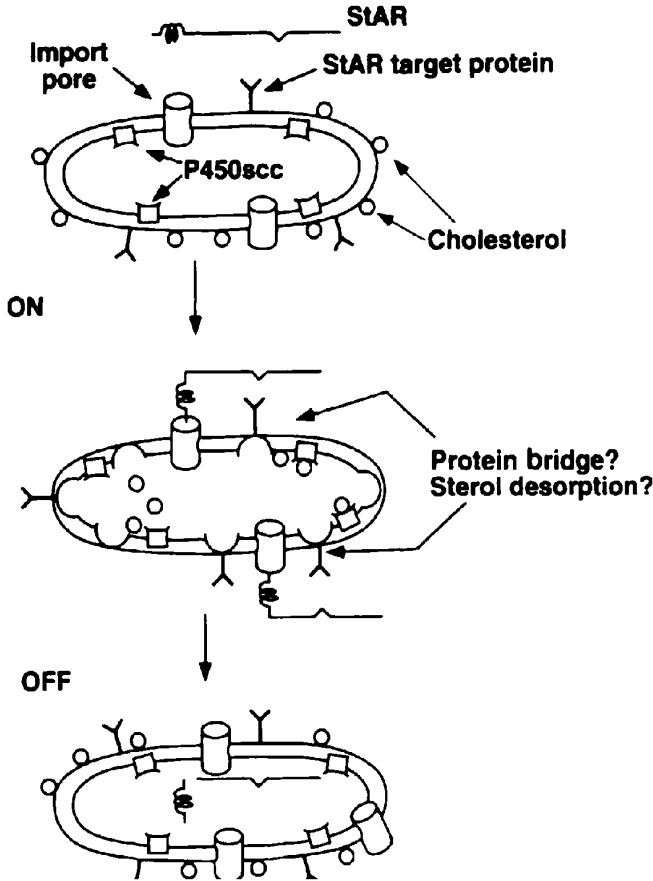
independent steroidogenesis prior to the severe cellular damage resulting from cholesterol accumulation and cholesterol oxidation, which ultimately results in a non-functional steroidogenic cell (Bose *et al.*, 1996). Comparison of the clinical course of 46, XX females with congenital lipoid adrenal hyperplasia to that of 46, XY subjects reinforced the pathophysiological mechanism proposed in Figure 5.2 (Bose *et al.*, 1997; Fujieda *et al.*, 1997). 46, XX females underwent spontaneous puberty and secondary sexual development, whereas 46, XY patients were unable to undergo spontaneous puberty and had insufficient testicular androgen production *in utero* to masculinize the external genitalia. The evident sparing of some ovarian function in the face of mutations that inactivate StAR reflects the presence of modest StAR-independent steroidogenesis in follicles that is sufficient to sponsor estradiol synthesis. Because the ovary is essentially steroidogenically quiescent during fetal life and because follicles only produce steroids when they are recruited to mature, most follicles are spared from the ravages of cholesterol engorgement, leaving a source of viable, albeit impaired, steroid-producing cells at the time of puberty.

StAR's role in steroidogenesis has been verified in mice made nullizygous for the StAR gene (Caron *et al.*, 1997). These mice exhibited a phenotype nearly identical to that of humans with congenital lipoid adrenal hyperplasia.

### Structure/function analysis of StAR

The molecular genetic studies of congenital lipoid adrenal hyperplasia suggested that the C-terminus of StAR was critical for steroidogenic activity. The N-terminal sequence of StAR is characteristic of mitochondrial targeting sequences. The presence of the mitochondrial targeting motif and cleavage sites for mitochondrial endoproteases in the rodent StAR protein, as well as the localization of StAR protein in putative contact sites between the outer and inner mitochondrial membranes, led to the initial model of StAR action. This model proposed that StAR formed contacts sites between the membranes through which cholesterol could flow down a chemical gradient as the protein was imported (Stocco and Clark, 1996; Cherradi *et al.*, 1997). Unexpectedly, StAR mutant proteins lacking the first 62 amino acids (N-62 mutant) which contain the mitochondrial targeting sequence were as effective as wild type StAR in stimulating steroidogenesis in the COS-1 cell steroidogenic enhancement bioassay (Arakane *et al.*, 1996, 1998b). The cytoplasmic distribution of the N-62 mutant StAR protein contrasted markedly with the almost exclusive intramitochondrial distribution of the wild type StAR. Also, truncation of 10 or more of the C-terminal amino acids markedly reduced StAR's steroidogenic activity.

The ability of StAR to stimulate steroidogenesis when it was localized to the cytoplasmic compartment suggested two different mechanisms of action: StAR could act on the outer surface of the mitochondria or, alternatively, could act in the cytoplasm on another protein. Extensive immunoprecipitation studies and yeast-two hybrid analyses failed to identify other proteins that could associate with the N-62 StAR molecule thus favoring an action of StAR on the outer mitochondrial membrane. Subsequent studies demonstrated that recombinant N-62 mutant protein could increase pregnenolone synthesis and the conversion of <sup>3</sup>H-cholesterol to <sup>3</sup>H-pregnenolone by isolated mitochondria (Arakane *et al.*, 1998a). In contrast, a mutant recombinant N-62 in which an alanine at the 218 amino acid was replaced



**Figure 5.3 A model of StAR action.** StAR, targeted to mitochondria by an N-terminal mitochondrial targeting sequence, acts on the outer mitochondrial membrane and transiently activates cholesterol transport to the inner mitochondrial membrane by promoting cholesterol desorption from the sterol-rich outer membrane to the sterol-poor inner membrane. Cholesterol may flow through proteinaceous bridges between the mitochondrial membranes (i.e., pre-existing contact sites) formed in response to StAR action. Cholesterol then serves as the substrate for P450<sub>scc</sub> (CYP11A), which catalyzes pregnenolone formation. Importation of StAR removes the protein from its site of action, the outer mitochondrial membrane, terminating cholesterol transport to the inner membrane. Cleavage of the StAR mitochondrial signal sequence following import into the mitochondria yields the mature 30 kDa protein.

with a valine was unable to stimulate steroidogenesis. The A218V mutation is found in patients with congenital lipoid adrenal hyperplasia (Bose *et al.*, 1996).

The ability of recombinant N-62 StAR to stimulate pregnenolone synthesis and the conversion of exogenous labeled cholesterol into labeled pregnenolone by isolated luteal

mitochondria suggests that StAR might be a sterol transfer protein. Kallen (1998), in fact, demonstrated that nanomolar concentrations of recombinant StAR were able to stimulate the transfer of cholesterol and the plant sterol,  $\beta$ -sitosterol, from sterol-rich-liposomes to heat-treated rat and mouse liver, bovine corpus luteum and yeast mitochondria in a dose-, time- and temperature dependent manner. These findings led to a model of StAR action (see Figure 5.3) in which the protein is efficiently directed to the mitochondria by the N-terminal mitochondrial targeting sequence. Prior to importation into the mitochondria, StAR ejects cholesterol from the outer membrane, causing it to move to the relatively sterol-poor inner membrane, perhaps at bridges between the membranes. StAR is then imported into the mitochondria and processed by metallo-endopeptidase to yield the mature form of the protein. By removing StAR from the outer mitochondrial membrane the steroidogenic action is terminated, thus accounting for the rapid action and short functional life of StAR and the requirement for continuous StAR synthesis to sustain steroidogenesis. Physical studies on recombinant StAR protein suggest it is a molten globule, a dynamic protein structure that lacks fixed interactions that would constrain the folded polypeptide chain organization (Rose *et al.*, 1999). The molten globule configuration appears at pH 3.5–4.0, a pH that may be generated in the immediate vicinity of mitochondria. The molten globule configuration may facilitate the unfolding of the protein in preparation for action on the mitochondria and the protein's subsequent movement through the import pore into the mitochondrial matrix.

The crystal structure of a StAR homologue (MLN 64, see below) was recently solved revealing that the StAR functional domain contains a hydrophobic tunnel capable of binding cholesterol. The capacity to associate with sterols may be critical to StAR's sterol transfer activity (Christensen and Strauss, 2000).

### Post-translational modifications of StAR: phosphorylation

StAR was originally identified as a phosphoprotein; subsequent analyses indicated that the protein contains putative phosphorylation sites for protein kinase A (PKA), CAM kinase, protein kinase C, creatine kinase and P34/CDC2 kinase. Comparison of the StAR protein sequences from multiple species has demonstrated two consensus motifs for protein kinase A (PKA) phosphorylation located at serine 57 and serine 195 in the human protein (Arakane *et al.*, 1997). Cyclic AMP is a known activator of PKA, qualifying these sites as potential candidates for modulation of StAR activity via PKA-mediated phosphorylation. Alkaline phosphatase treatment of StAR-expressing COS-1 cell lysates resulted in loss of two acidic species noted by two dimensional gel electrophoresis (Arakane *et al.*, 1997). Moreover,  $^{32}\text{P}$  was incorporated into StAR protein immunoprecipitated from COS-1 cell extracts, and treatment with 8-bromo-cAMP increased  $^{32}\text{P}$  incorporation into the StAR preprotein. StAR protein generated by *in vitro* transcription/translation was phosphorylated by the protein kinase A catalytic subunit in the presence of [ $\gamma$ - $^{32}\text{P}$ ] ATP.  $^{32}\text{P}$  incorporation was diminished by mutation of these serine residues to alanines, both in COS-1 cell transfection experiments and using protein generated by *in vitro* transcription/translation incubated with the PKA catalytic subunit (Arakane *et al.*, 1997). These data are consistent with a role for PKA in StAR phosphorylation.



To assess the functional significance of StAR phosphorylation at these residues, the steroidogenic activity of the wild-type StAR and serine to alanine mutants was tested in COS-1 cells transfected with the cholesterol side-chain cleavage enzyme system. Mutation of the conserved PKA target site at serine 57 had no effect on pregnenolone synthesis, while mutation at residue 195 resulted in approximately 50% reduction in steroid production. Mutation of serine 195 to an aspartic acid resulted in slightly increased StAR activity in the COS-1 cells, consistent with the notion that negative charge at this site, via phosphorylation or otherwise, enhances StAR function. These observations suggest that phosphorylation of serine 195 increases the biological activity of StAR and that this post- or co-translational event accounts, in part, for the immediate effects of cAMP on steroid production (Arakane *et al.*, 1997).

### StAR expression and StAR homologues

Steroidogenic activity in adrenal and gonadal cells is directly correlated with the presence of a major ~1.6kb StAR mRNA transcript with lesser transcripts of 4.4 and 7.5kb (Kiriakidou *et al.*, 1996; Stocco and Clark, 1996). Additionally, the ~1.6kb transcript is expressed at low levels in the kidney (site of Vitamin D 1 $\alpha$ -hydroxylation) (Sugawara *et al.*, 1995a). StAR mRNA transcripts are not detected in human placental tissues which is consistent with the observation that placental steroidogenesis is StAR-independent. In accordance with this finding, pregnancies hosting fetuses affected with congenital lipoid adrenal hyperplasia progress to term, demonstrating adequate placental progesterone production despite the fact that the placenta is an organ of fetal origin.

The placental production of progesterone may be facilitated by the existence of a StAR-like protein in this organ, which subserves StAR's function. MLN64, first identified as a gene highly expressed in certain breast cancers, is also expressed in the placenta and brain, two tissues that produce steroid hormones but do not contain detectable StAR mRNA (Watari *et al.*, 1997). MLN64 was found to have a carboxy terminus with strong homology to StAR. Moreover, MLN64 sequences corresponding to essential domains in StAR were found to stimulate steroidogenesis in COS cells co-transfected with the P450<sub>scc</sub> enzyme system. Western blot analysis revealed that MLN64 fragments representing domains of MLN64 most similar to StAR and displaying the greatest Steroidogenic activity in COS cells were present in placenta and choriocarcinoma cells (Watari *et al.*, 1997). Thus, a family of StAR-like proteins exist that are expressed in a tissue-specific manner and are differentially regulated, promoting steroidogenesis in diverse cell types.

### Transcriptional regulation

Cyclic AMP analogues stimulate StAR gene transcription by a process that requires ongoing protein synthesis (Kiriakidou *et al.*, 1996). The StAR promoter, like several other Steroidogenic enzyme gene promoters, lacks cAMP-responsive elements (CRE) (Waterman, 1994; Sugawara *et al.*, 1995b). The cyclic AMP-responsiveness of the StAR promoter was shown to be conferred by Steroidogenic factor-1 (SF-1/AdBP4), an orphan member of the nuclear hormone receptor superfamily of transcription factors (Parker and

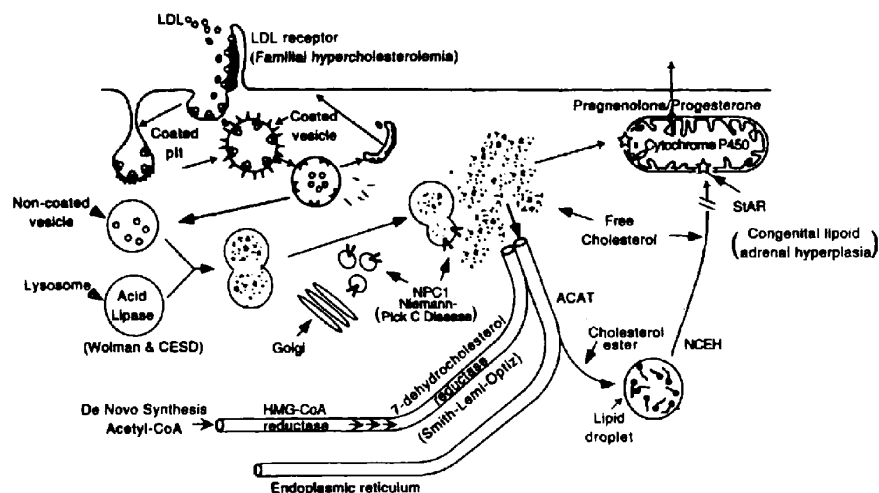
Schimmer, 1997). SF-1 binding sites are present in the promoters of many of the genes encoding steroidogenic enzymes and they have been shown to confer cAMP-responsiveness to these promoters. SF-1 was shown to render the StAR promoter cAMP-responsive in the context of cells that do not express endogenous SF-1 (i.e., BeWo choriocarcinoma cells, HeLa cells, and SK-OV-3 cells) (Sugawara *et al.*, 1996).

Three SF-1 binding elements in the 5'-flanking region of the StAR gene have been identified (Sugawara *et al.*, 1996, 1997). These response elements were protected by SF-1 in DNase 1 footprint analysis and bound GST-SF-1 fusion protein in electrophoretic mobility shift assays. The two proximal SF-1 response elements were shown to be critical for StAR promoter function in human granulosa-lutein cells, cells which express SF-1 and respond to cAMP with increased transcription of the endogenous StAR gene. Mutation of either element substantially reduced basal and forskolin-stimulated promoter activity. Mutation of the distal SF-1 binding site reduced basal but not forskolin-stimulated promoter activity in the granulosa-lutein cells. These findings demonstrated that multiple SF-1 response elements are required for maximal StAR promoter activity and for regulation by cAMP. The mechanism by which SF-1 confers cAMP-responsiveness at each of these sites, and the potential regulators of SF-1 function (i.e., ligands, phosphorylation, other protein cofactors) are areas of intense investigation (Lala *et al.*, 1997; Christenson *et al.*, 1998; Crawford *et al.*, 1998; Ito *et al.*, 1998; Sadovsky and Crawford, 1998; Hammer *et al.*, 1999).

The human StAR gene promoter also contains two cis elements that are responsive to CCAAT/enhancer binding proteins (C/EBPs) (Christenson *et al.*, 1999). One of these elements was shown to bind C/EBP $\beta$  present in granulosa lute in cell nuclear extracts. When this site was mutated, basal StAR promoter activity in granulosa-lutein cells was reduced, but the mutated promoter still showed a twofold increase in activity in response to 8-Br-cAMP. 8-Br-cAMP treatment was also shown to increase granulosa-lutein cell C/EBP $\beta$  levels, explaining in part the requirement for ongoing protein synthesis for cAMP-induced StAR gene transcription. Therefore, part of the augmentation of StAR gene expression by trophic hormones encompasses signaling mediated by C/EBP $\beta$  as well as the action of SF-1.

A role for Sp1 response elements in the transactivation of the StAR promoter in response to cAMP can also be envisioned. Several consensus Sp1 sites are located in the human StAR promoter and Sp1 response elements are known to be important in conferring cAMP-responsiveness to other steroidogenic enzyme promoters (Momoi *et al.*, 1992; Sugawara *et al.*, 1995b). It is noteworthy that Sp1 acts as a cofactor for the sterol regulatory element binding proteins (SREBPs), a family of ubiquitously expressed transcription factors essential for the regulation of many proteins important in the synthesis and metabolism of lipids. Co-expression of SREBP-1a has been found to increase StAR promoter activity (Christenson *et al.*, 1998).

Another member of the nuclear hormone receptor superfamily, DAX-1, has been implicated in the negative regulation of StAR gene expression. Mutations in the human DAX-1 gene cause X-linked adrenal hypoplasia congenita. DAX-1 has been shown to repress both basal and cAMP-induced StAR promoter activity (Zazopoulos *et al.*, 1997). DAX-1 may inhibit StAR gene expression by binding to SF-1 or by directly binding to the StAR promoter.



**Figure 5.4 Overview of cellular cholesterol metabolism.** The movement of cholesterol is tracked in a steroidogenic cell. The human genetic diseases that allowed the dissection of this pathway are listed next to their respective gene products that govern cholesterol dynamics. ACAT, acyl-CoA cholesterol acyl transferase; NPC, Niemann-Pick type C1 protein; NCEH, neutral cholesterol ester hydrolase; StAR, steroidogenic acute regulatory protein.

## CONCLUSIONS

Figure 5.4 illustrates the complex itineraries taken by cholesterol derived from lipoproteins and *de novo* synthesis as it enters into a pool used for the steroid synthesis. The search for the protein(s) involved in cholesterol trafficking and regulation of cholesterol content within specific membranes has been, and will likely remain, particularly a difficult task because of the lack of facile methods to identify and trace cholesterol movement in cells, the appearance of cholesterol in most membranes and the multiple methods of acquiring cholesterol (i.e., *de novo* synthesis, selective uptake, receptor-mediated uptake). Thus, the study of human genetic diseases and their molecular characterization has been instrumental in obtaining significant insight into the regulatory mechanisms involved in cholesterol uptake, biosynthesis, storage, and intracellular trafficking. In fact, in many cases the study of the disease process was pivotal for the isolation and cloning of the genes involved in cholesterol metabolic pathways (e.g., familial hypercholesterolemia-LDL receptor, Niemann-Pick C disease-NPC1 protein). In other instances, molecular genetic studies confirmed the importance of a known gene product by demonstrating its involvement in a disease process (e.g., StAR-congenital lipid adrenal hyperplasia).

Furthermore, the molecular genetic study of congenital lipid adrenal hyperplasia provided the identification of the important structural motifs in the StAR molecule. While significant progress has been made and a preliminary model of StAR action has emerged, a number of important questions remain. Answers to these questions will provide new insight

into the regulation of steroidogenesis, as well as the general mechanisms underlying intracellular cholesterol trafficking.

## REFERENCES

- Acton, S., Rigotti, A., Landschulz, K.T., Xu, S., Hobbs, H.H. and Krieger, M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271**, 518–520.
- Acton, S.L., Scherer, P.E., Lodish, H.F. and Krieger, M. (1994) Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J. Biol. Chem.* **269**, 21003–21009.
- Almabobi, G., Williams, L.J. and Hall, P.P. (1992a) Attachment of mitochondria to intermediate filaments in adrenal cells: relevance to the regulation of steroid synthesis. *Exp. Cell Res.* **200**, 361–369.
- Almabobi, G., Williams, L.J. and Hall, P.P. (1992b) Attachment of steroidogenic lipid droplets to intermediate filaments in adrenal cells. *J. Cell Sci.* **101**, 383–393.
- Anderson, R.A., Byrum, R.S., Coates, P.M. and Sando, G.N. (1994) Mutations at the lysosomal acid cholesteryl ester hydrolase gene locus in Wolman disease. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2718–2722.
- Anderson, R.A. and Sando, G.N. (1991) Cloning and expression of cDNA encoding human lysosomal acid lipase/cholesteryl ester hydrolase: similarities to gastric and lingual lipases. *J. Biol. Chem.* **66**, 22479–22484.
- Andersson, H.C., Frentz, J., Martinez, J.E., Tuck-Muller, C.M. and Bellizaire, J. (1999) Adrenal insufficiency in Smith-Lemli-Opitz syndrome. *Am. J. Med. Genet.* **82**, 382–384.
- Arakane, P., Kallen, C.B., Watari, H., Foster, J.A., Sepuri, N.B., Pain, D., Stayrook, S.E., Lewis, M., Gerton, G.L. and Strauss, III, J.F. (1998a) The mechanism of action of steroidogenic acute regulatory protein (StAR): StAR acts on the outside of mitochondria to stimulate steroidogenesis. *J. Biol. Chem.* **3**, 16339–16345.
- Arakane, F., Kallen, C.B., Watari, H., Stayrook, S.E., Lewis, M. and Strauss, III, J.F. (1998b) Steroidogenic acute regulatory protein (StAR) acts on the outside of mitochondria to stimulate steroidogenesis. *Endocr. Res.* **24**, 463–468.
- Arakane, F., King, S.R., Du, Y., Kallen, C.B., Walsh, L.P., Watari, H., Stocco, D.M. and Strauss, III, J.F. (1997) Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *J. Biol. Chem.* **272**, 32656–32662.
- Arakane, F., Sugawara, T., Nishino, H., Liu, Z., Holt, J.A., Pain, D., Stocco, D.M., Miller, W.L. and Strauss, III, J.F. (1996) Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: implications for the mechanism of StAR action. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13731–13736.
- Aslanidis, C., Klima, H., Lackner, K.J. and Schmitz, G. (1994) Genomic organization of the human lysosomal acid lipase gene (LIPA). *Genomics* **20**, 329–331.
- Assmann, G. and Sedorf, U. (1995) Acid lipase deficiency: Wolman disease and cholesteryl ester storage disease. In: *The Metabolic and Molecular Bases of Inherited Disease*, C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle (eds), Vol. 2, McGraw-Hill Inc., New York, pp. 2563–2588.
- Aten, R.F., Kolodecik, T.R., MacDonald, G.J. and Behrman, H.R. (1995) Modulation of cholesteryl ester hydrolase messenger ribonucleic acid levels, protein levels, and activity in the rat corpus luteum. *Biol. Reprod.* **53**, 1110–1117.

- Azhar, S., Luo, Y., Medicherla, S. and Reaven, E. (1999) Upregulation of selective cholesteryl ester uptake pathway in mice with deletion of low-density lipoprotein receptor function. *J. Cell. Physiol.* **180**, 190–202.
- Belloni, A.S., Mazzocchi, G., Mantero, F. and Nussdorfer, G.G. (1987) The human adrenal cortex: ultrastructure and base-line morphometric data. *J. Submicr. Cytol.* **19**, 657–668.
- Boizel, R., de Peretti, E., Cathiard, A.M., Halimi, S., Bost, M., Berthezene, F. and Saez, J.M. (1986) Pattern of plasma levels of cortisol, dehydroepiandrosterone and pregnenolone sulphate in normal subjects and in patients with homozygous familial hypercholesterolaemia during ACTH infusion. *Clin. Endocrinol. (Oxford)* **25**, 363–371.
- Bose, H.S., Baldwin, M.A. and Miller, W.L. (1998) Incorrect folding of steroidogenic acute regulatory protein (StAR) in congenital lipid adrenal hyperplasia. *Biochemistry* **37**, 9768–9775.
- Bose, H.S., Pescovitz, O.H. and Miller, W.L. (1997) Spontaneous feminization in a 46, XX female patient with congenital lipid adrenal hyperplasia due to a homozygous frameshift mutation in the steroidogenic acute regulatory protein. *J. Clin. Endocrinol. Metab.* **82**, 1511–1515.
- Bose, H.S., Sugawara, T., Strauss, III, J.F. and Miller, W.L. (1996) The pathophysiology and genetics of congenital lipid adrenal hyperplasia. International Congenital Lipoid Adrenal Hyperplasia Consortium. *N. Eng. J. Med.* **335**, 1870–1878.
- Bose, H.S., Whittall, R.M., Baldwin, M.A. and Miller, W.L. (1999) The active form of the steroidogenic acute regulatory protein, StAR, appears to be a molten globule. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7250–7255.
- Caron, K.M., Soo, S.C., Wetsel, W.C., Stocco, D.M., Clark, B.J. and Parker, K.L. (1997) Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipid adrenal hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11540–11545.
- Carr, B.R. and Simpson, E.R. (1981) Lipoprotein utilization and cholesterol synthesis by the human fetal adrenal gland. *Endocr. Rev.* **2**, 306–326.
- Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., *et al.*, (1997) Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* **277**, 228–231.
- Chasalow, F.I., Blethen, S.L. and Taysi, K. (1985) Possible abnormalities of steroid secretion in children with Smith-Lemli-Opitz syndrome and their parents. *Steroids* **46**, 827–843.
- Cherradi, N., Rossier, M.F., Vallotton, M.B., Timberg, R., Friedberg, I., Orly, J., Wang, X.J., Stocco, D.M. and Capponi, A.M. (1997) Submitochondrial distribution of three key steroidogenic proteins (steroidogenic acute regulatory protein and cytochrome P450<sub>scc</sub> and 3 $\beta$ -hydroxy steroid dehydrogenase isomerase enzymes) upon stimulation by intracellular calcium in adrenal glomerulosa cells. *J. Biol. Chem.* **272**, 7899–7907.
- Christenson, L.K., Johnson, P.F., McAllister, J.M. and Strauss, III, J.F. (1999) CCAAT/Enhancer-binding proteins regulate expression of the human steroidogenic acute regulatory protein (StAR) gene. *J. Biol. Chem.* **274**, 26591–26598.
- Christenson, L.K., McAllister, J.M., Martin, K.O., Javitt, N.B., Osborne, T.F. and Strauss, III, J.F. (1998) Oxysterol regulation of steroidogenic acute regulatory protein gene expression. Structural specificity and transcriptional and posttranscriptional actions. *J. Biol. Chem.* **273**, 30729–30735.
- Christensen, L.K. and Strauss, III, J.F. (2000) Steroidogenic acute regulatory protein (StAR) and the intramitochondrial translocation of cholesterol. *Biochim. Biophys. Acta* **1529**, 175–187.
- Chung, S., Wang, S.P., Pan, L., Mitchell, G., Trasler, J. and Hermo, L. (2001) Infertility and testicular defects in hormone-sensitive lipase-deficient mice. *Endocrinology* **142**, 4272–4281.
- Clark, B.J., Wells, J., King, S.R. and Stocco, D.M. (1994) The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse

- Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR) . *J. Biol. Chem.* **269**, 28314–28322.
- Colucci-Guyon, E., Portier, M.M., Dunia, I., Paulin, D., Pournin, S. and Babinet, C. (1994) Mice lacking vimentin develop and reproduce without an obvious phenotype. *Cell* **79**, 679–694.
- Crawford, P.A., Dorn, C., Sadovsky, Y. and Milbrandt, J. (1998) Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. *Mol. Cell Biol.* **18**, 2949–2956.
- Davies, J.P., Chen, F.W. and Ioannou, Y.A. (2000) Transmembrane molecular pump activity of Niemann-Pick C1 protein. *Science* **290**, 2295–2298.
- Deeb, S.S., Distèche, C., Motulsky, A.G., Lebo, R.V. and Kan, Y.W. (1986) Chromosomal localization of the human apolipoprotein B gene and detection of homologous RNA in monkey intestine. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 419–422.
- Degenhart, H.J., Visser, H.K., Boon, H. and O'Doherty, N.J. (1972) Evidence for deficient 20 $\alpha$  cholesterol-hydroxylase activity in adrenal tissue of a patient with lipoid adrenal hyperplasia . *Acta Endocrinol. (Copenh)*. **71**, 512–518.
- Du, H., Duanmu, M., Witte, D. and Grabowski, G.A. (1998) Targeted disruption of the mouse lysosomal acid lipase gene: long-term survival with massive cholesteryl ester and triglyceride storage. *Hum. Mol. Genet.* **7**, 1347–1354.
- Enders, A.C. (1973) Cytology of the corpus luteum. *Biol. Reprod.* **8**, 158–182.
- Farese, Jr., R.V. (1998) Acyl CoA: cholesterol acyltransferase genes and knockout mice. *Curr. Opin. Lipidol.* **9**, 119–123.
- Farese, Jr., R.V., Linton, M.F. and Young, S.G. (1992) Apolipoprotein B gene mutations affecting cholesterol levels. *J. Intern. Med.* **231**, 643–652.
- Farnsworth, W.H., Hoeg, J.M., Maher, M., Brittain, E.H., Sherins, R.J. and Brewer, Jr., H.B. (1987) Testicular function in type II hyperlipoproteinemic patients treated with lovastatin (mevinolin) or neomycin. *J. Clin. Endocrinol. Metab* **65**, 546–550.
- Ferguson, J.J. (1962) Puromycin and adrenal-responsiveness to adrenocorticotrophic hormone. *Biochim. Biophys. Acta* **57**, 616–617.
- Ferguson, J.J. (1963) Protein synthesis and adrenocorticotropin responsiveness. *J. Biol. Chem.* **238**, 2754–2759.
- Fidge, N.H. (1999) High density lipoprotein receptors, binding proteins, and ligands. *J. Lipid Res.* **40**, 187–201.
- Fielding, C.J. and Fielding, P.E. (1997) Intracellular cholesterol transport. *J. Lipid Res.* **38**, 1503–1521.
- Fitoussi, G., Negre-Salvayre, A., Pieraggi, M.T. and Salvayre, R. (1994) New pathogenetic hypothesis for Wolman disease: possible role of oxidized low-density lipoproteins in adrenal necrosis and calcification. *Biochem. J.* **301**, 267–273.
- FitzPatrick, D.R., Keeling, J.W., Evans, M.J., Kan, A.E., Bell, J.E., Porteous, M.E., Mills, K., Winter, R.M. and Clayton, P.T. (1998) Clinical phenotype of desmosterolosis. *Am. J. Med. Genet.* **75**, 145–152.
- Fujieda, K., Tajima, T., Nakae, J., Sageshima, S., Tachibana, K., Suwa, S., Sugawara, T. and Strauss, III, J.F. (1997) Spontaneous puberty in 46, XX subjects with congenital lipoid adrenal hyperplasia. Ovarian steroidogenesis is spared to some extent despite inactivating mutations in the steroidogenic acute regulatory protein (StAR) gene. *J. Clin. Invest.* **99**, 1265–1271.
- Goldstein, J.L., Hobbs, H.H. and Brown, M.S. (1995) Familial hypercholesterolemia. In: *The Metabolic and Molecular Bases of Inherited Disease*, C.R.Scriver, A.L.Beaudet, W.S.Sly and D.Valle (eds), McGraw-Hill Inc., New York, pp. 1981–2030.
- Golos, T.G. and Strauss, III, J.F. (1988) 8-bromoadenosine cyclic 3', 5'-phosphate rapidly increases 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA in human granulosa cells: role of

- cellular sterol balance in controlling the response to tropic stimulation. *Biochemistry* **27**, 3503–3506.
- Gwynne, J.T. and Strauss, III, J.F. (1982) The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr. Rev.* **3**, 299–329.
- Haddad, L., Day, I.N., Hunt, S., Williams, R.R., Humphries, S.E. and Hopkins, P.N. (1999) Evidence for a third genetic locus causing familial hypercholesterolemia. A non-LDLR, non-APOB kindred. *J. Lipid Res.* **40**, 1113–1122.
- Hall, P.F. (1986) Cytochromes P-450 and the regulation of steroid synthesis. *Steroids* **48**, 131–196.
- Hall, P.F. and Almabobi, G. (1997) Roles of microfilaments and intermediate filaments in adrenal steroidogenesis. *Micr. Res. Techniques* **36**, 463–479.
- Hammer, G.D., Krylova, I., Zhang, Y., Darimont, B.D., Simpson, K., Weigel, N.L. and Ingraham, H.A. (1999) Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. *Mol. Cell* **3**, 521–526.
- Hauffa, B.P., Miller, W.L., Grumbach, M.M., Conte, F.A. and Kaplan, S.L. (1985) Congenital adrenal hyperplasia due to deficient cholesterol side-chain cleavage activity (20, 22-desmolase) in a patient treated for 18 years. *Clin. Endocrinol. (Oxford)* **23**, 481–493.
- Higgins, M.E., Davies, J.P., Chen, F.W. and Ioannou, Y.A. (1999) Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. *Mol. Genet. Metab.* **68**, 1–13.
- Illingworth, D.R., Corbin, D.K., Kemp, E.D. and Keenan, E.J. (1982a) Hormone changes during the menstrual cycle in abetalipoproteinemia: reduced luteal phase progesterone in a patient with homozygous hypobetalipoproteinemia. *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6685–6689.
- Illingworth, D.R., Kenny, T.A. and Orwoll, E.S. (1982b) Adrenal function in heterozygous and homozygous hypobetalipoproteinemia. *J. Clin. Endocrinol. Metab.* **54**, 27–33.
- Illingworth, D.R., Lees, A.M. and Lees, R.S. (1983) Adrenal cortical function in homozygous familial hypercholesterolemia. *Metabolism* **32**, 1045–1052.
- Innerarity, T.L., Mahley, R.W., Weisgraber, K.H., Bersot, T.P., Krauss, R.M., Vega, G.L., Grundy, S.M., Friedl, W., Davignon, J. and McCarthy, B.J. (1990) Familial defective apolipoprotein B-100: a mutation of apolipoprotein B that causes hypercholesterolemia. *J. Lipid Res.* **31**, 1337–1349.
- Ito, M., Yu, R.N. and Jameson, J.L. (1998) Steroidogenic factor-1 contains a carboxy-terminal transcriptional activation domain that interacts with steroid receptor coactivator-1. *Mol. Endocrinol.* **12**, 290–301.
- Iwamoto, K., Yang, X., Rogerson, F.M., Mason, J.I., Artwohl, J., Bolin, K., Klimah, P., Swart, P. and Pang, S. (1994) Evidence of a Steroidogenic enzyme gene dose effect on adrenal gene expression in hereditary rabbit congenital adrenal hyperplasia. *Ped. Res.* **36**, 660–666.
- Jacobs, N.L., Andemariam, B., Underwood, K.W., Panchalingam, K., Sternberg, D., Kielian, M. and Liscum, L. (1997) Analysis of a Chinese hamster ovary cell mutant with defective mobilization of cholesterol from the plasma membrane to the endoplasmic reticulum. *J. Lipid Res.* **38**, 1973–1987.
- Jay, R.H., Sturley, R.H., Stirling, C., McGarrigle, H.H., Katz, M., Reckless, J.P. and Betteridge, D.J. (1991) Effects of pravastatin and cholestyramine on gonadal and adrenal steroid production in familial hypercholesterolaemia. *Br. J. Clin. Pharmacol.* **32**, 417–422.
- Kallen, C.B., Billheimer, J.T., Summers, S.A., Stayrook, S.E., Lewis, M. and Strauss, III, J.F. (1998) Steroidogenic acute regulatory protein (StAR) is a sterol transfer protein. *J. Biol. Chem.* **273**, 26285–26288.

- Kiriakidou, M., McAllister, J.M., Sugawara, T. and Strauss, III, J.F. (1996) Expression of Steroidogenic acute regulatory protein (StAR) in the human ovary. *J. Clin. Endocrinol. Metab.* **81**, 4122–4128.
- Kraemer, F.B., Patel, S., Saedi, M.S. and Sztalryd, C. (1993) Detection of hormone-sensitive lipase in various tissues. I. Expression of an HSL/bacterial fusion protein and generation of anti-HSL antibodies. *J. Lipid Res.* **34**, 663–671.
- Lala, D.S., Syka, P.M., Lazarchik, S.B., Mangelsdorf, D.J., Parker, K.L. and Heyman, R.A. (1997) Activation of the orphan nuclear receptor Steroidogenic factor 1 by oxysterols. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4895–4900.
- Lange, Y., Strebel, F. and Steck, T.L. (1993) Role of the plasma membrane in cholesterol esterification in rat hepatoma cells. *J. Biol. Chem.* **268**, 13838–13843.
- Laue, L., Hoeg, J.M., Barnes, K., Loriaux, D.L. and Chrousos, G.P. (1987) The effect of mevinolin on steroidogenesis in patients with defects in the low density lipoprotein receptor pathway. *J. Clin. Endocrinol. Metab.* **64**, 531–535.
- Lin, D., Gitelman, S.E., Saenger, P. and Miller, W.L. (1991) Normal genes for the cholesterol side chain cleavage enzyme, P450scc, in congenital lipid adrenal hyperplasia. *J. Clin. Invest.* **88**, 1955–1962.
- Lin, D., Sugawara, T., Strauss, III, J.F., Clark, B.J., Stocco, D.M., Saenger, P., Rogol, A. and Miller, W.L. (1995) Role of Steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267**, 1828–1831.
- Liscum, L. and Munn, N.J. (1999) Intracellular cholesterol transport. *Biochim. Biophys. Acta* **1438**, 19–37.
- Liscum, L. and Underwood, K.W. (1995) Intracellular cholesterol transport and compartmentation. *J. Biol. Chem.* **270**, 15443–15446.
- Loftus, S.K., Morris, J.A., Carstea, E.D., Gu, J.Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M.A., Tagle, D.A., Pentchev, P.G. and Pavan, W.J. (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science* **277**, 232–235.
- Lohse, P., Maas, S., Sewell, A.C., van Diggelen, O.P. and Seidel, D. (1999) Molecular defects underlying Wolman disease appear to be more heterogeneous than those resulting in cholesteryl ester storage disease. *J. Lipid Res.* **40**, 221–228.
- Londos, C., Brasaemle, D.L., Gruia-Gray, J., Servetnick, D.A., Schultz, C.J., Levin, D.M. and Kimmel, A.R. (1995) Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and Steroidogenic cells. *Biochem. Soc. Trans.* **23**, 611–615.
- Londos, C., Brasaemle, D.L., Schultz, C.J., Segrest, J.P. and Kimmel, A.R. (1999) Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Sem. Cell Dev. Biol.* **10**, 51–58.
- Ludwig, E.H., Hopkins, P.N., Allen, A., Wu, L.L., Williams, R.R., Anderson, J.L., Ward, R.H., Lalouel, J.M. and Innerarity, T.L. (1997) Association of genetic variations in apolipoprotein B with hypercholesterolemia, coronary artery disease, and receptor binding of low density lipoproteins. *J. Lipid Res.* **38**, 1361–1373.
- Mannella, C.A., Hsieh, C.E. and Marko, M. (1999) Electron microscopic tomography of whole, frozen-hydrated rat liver mitochondria at 400 KV. In: *Proceedings of Microscopy and Microanalysis*. G.W. Bailey, W.G. Jerome, S. McKernan, J.F. Mansfield and R.L. Price (eds), Springer-Verlag, New York, pp. 416–417.
- McKeever, P.A. and Young, I.D. (1990) Smith-Lemli-Opitz syndrome. II: A disorder of the fetal adrenals? *J. Med. Genet.* **27**, 465–466.
- Meiner, V.L., Cases, S., Myers, H.M., Sande, E.R., Bellosta, S., Schambelan, M., Pitas, R.E., McGuire, J., Herz, J. and Farese, Jr., R.V. (1996) Disruption of the acyl-CoA: cholesterol



- acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14041–14046.
- Miller, W.L. and Strauss, III, J.F. (1999) Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StAR. *J. Steroid Biochem. Mol. Biol.* **69**, 131–141.
- Moebius, F.F., Fitzky, B.U. and Glossmann, H. (2000) Genetic defects in postsqualene cholesterol biosynthesis. *Trends Endocrinol. Metab.* **11**, 106–114.
- Momoi, K., Waterman, M.R., Simpson, E.R. and Zanger, U.M. (1992) 3', 5'-cyclic adenosine mono-phosphate-dependent transcription of the CYP11A (cholesterol side chain cleavage cytochrome P450) gene involves a DNA response element containing a putative binding site for transcription factor Spl. *Mol. Endocrinol.* **6**, 1682–1690.
- Morris, J.A., Zhang, D., Coleman, K.G., Nagle, J., Pentchev, P.G. and Carstea, E.D. (1999) The genomic organization and polymorphism analysis of the human Niemann-Pick C1 gene. *Biochem. Biophys. Res. Commun.* **261**, 493–498.
- Murao, K., Terpstra, V., Green, S.R., Kondratenko, N., Steinberg, D. and Quehenberger, O. (1997) Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *J. Biol. Chem.* **272**, 17551–17557.
- Myant, N.B. (1993) Familial defective apolipoprotein B-100: a review, including some comparisons with familial hypercholesterolaemia [published erratum appears in *Atherosclerosis* 1994 Feb; 105 (2):253]. *Atherosclerosis* **104**, 1–18.
- Nakae, J., Tajima, T., Sugawara, T., Arakane, F., Hanaki, *et al.* (1997) Analysis of the steroidogenic acute regulatory protein (StAR) gene in Japanese patients with congenital lipoid adrenal hyperplasia. *Hum. Mol. Genet.* **6**, 571–576.
- Naureckiene, S., Sleat, D.E., Lackland, H. *et al.* (2000) Identification of HEI as the second gene of Niemann-Pick's disease. *Science* **290**, 2298–2301.
- Nestler, J.E., Takagi, K. and Strauss, III, J.F. (1992) Lipoprotein and Cholesterol metabolism in cells that synthesize steroid hormones. In: *Advances in Cholesterol Research*. M.Esfahani and J.B.Swaney (eds), The Telford Press, pp. 133–169.
- Neufeld, E.B., Wastney, M., Patel, S., Suresh, S., Cooney, *et al.*, (1999) The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J. Biol. Chem.* **274**, 9627–9635.
- Nussdorfer, G.G. (1986) Cytophysiology of the adrenal cortex. *Int. Rev. Cytol.* **98**, 1–405.
- Ohno, Y., Yanagibashi, K., Yonezawa, Y., Ishiwatari, S. and Matsuba, M. (1983) A possible role of "steroidogenic factor" in the corticoidogenic response to ACTH; effect of ACTH, cycloheximide and aminoglutethimide on the content of cholesterol in the outer and inner mitochondrial membrane of rat adrenal cortex. *Endocrinol. Jap.* **30**, 335–338.
- Okuyama, E., Nishi, N., Onishi, S., Itoh, S., Ishii, Y., Miyataka, H., Fujita, K. and Ichikawa, Y. (1997) A novel splicing junction mutation in the gene for the steroidogenic acute regulatory protein causes congenital lipoid adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **82**, 2337–2342.
- Opitz, J.M. (1994) RSH/SLO ("Smith-Lemli-Opitz") syndrome: historical, genetic, and developmental considerations. *Am. J. Med. Genet.* **50**, 344–346.
- Osborne, T.F. (1995) Transcriptional control mechanisms in the regulation of cholesterol balance. *Critical Reviews in Eukaryotic Gene Expression* **5**, 317–335.
- Paavola, L.G., Strauss, III, J.F., Boyd, C.O. and Nestler, J.E. (1985) Uptake of gold- and [3H]cholesteryl linoleate-labeled human low density lipoprotein by cultured rat granulosa cells: cellular mechanisms involved in lipoprotein metabolism and their importance to steroidogenesis. *J. Cell Biol.* **100**, 1235–1247.

- Pagani, F., Pariyarath, R., Garcia, R., Stuani, C., Burlina, A.B., Ruotolo, G., Rabusin, M. and Baralle, F.E. (1998) New lysosomal acid lipase gene mutants explain the phenotype of Wolman disease and cholesteryl ester storage disease. *J. Lipid Res.* **39**, 1382–1388.
- Pang, S., Yang, X., Wang, M., Tissot, R., Nino, M., Manaligod, J., Bullock, L.P. and Mason, J.I. (1992) Inherited congenital adrenal hyperplasia in the rabbit: absent cholesterol side-chain cleavage cytochrome P450 gene expression. *Endocrinology* **131**, 181–186.
- Parker, Jr., C.R., Illingworth, D.R., Bissonnette, J. and Carr, B.R. (1986) Endocrine changes during pregnancy in a patient with homozygous familial hypobetalipoproteinemia. *N. Eng. J. Med.* **314**, 557–560.
- Parker, K.L. and Schimmer, B.P. (1997) Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr. Rev.* **18**, 361–377.
- Pentchev, P.G., Blanchette-Mackie, E.J. and Liscum, L. (1997) Biological implications of the Niemann-Pick C mutation. *Subcell. Biochem.* **28**, 437–451.
- Perides, G., Harter, C. and Traub, P. (1987) Electrostatic and hydrophobic interactions of the intermediate filament protein vimentin and its amino terminus with lipid bilayers. *J. Biol. Chem.* **262**, 13742–13749.
- Plump, A.S., Erickson, S.K., Weng, W., Partin, J.S., Breslow, J.L. and Williams, D.L. (1996) Apolipoprotein A-I is required for cholesteryl ester accumulation in Steroidogenic cells and for normal adrenal steroid production. *J. Clin. Invest.* **97**, 2660–2671.
- Privalle, C.T., Crivello, J.F. and Jefcoate, C.R. (1983) Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 702–706.
- Pullinger, C.R., Hennessy, L.K., Chatterton, J.E., Liu, W., Love, J.A., Mendel, C.M., Frost, P.H., Malloy, M.J., Schumaker, V.N. and Kane, J.P. (1995) Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. *J. Clin. Invest.* **95**, 1225–1234.
- Reinhart, M.P., Billheimer, J.T., Faust, J.R. and Gaylor, J.L. (1987) Subcellular localization of the enzymes of cholesterol biosynthesis and metabolism in rat liver. *J. Biol. Chem.* **262**, 9649–9655.
- Richards, J.S. (1994) Hormonal control of gene expression in the ovary. *Endocr. Rev.* **15**, 725–751.
- Rigotti, A., Trigatti, B.L., Penman, M., Rayburn, H., Herz, J. and Krieger, M. (1997) A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12610–12615.
- Roff, C.F., Strauss, III, J.F., Goldin, E., Jaffe, H., Patterson, M.C., Agritellis, G.C., Hibbs, A.M., Garfield, M., Brady, R.O. and Pentchev, P.G. (1993) The murine Niemann-Pick type C lesion affects testosterone production. *Endocrinology* **133**, 2913–2923.
- Rudel, L.L., Lee, R.G. and Cockman, T.L. (2001) Acyl coenzyme A: cholesterol acyltransferase types 1 and 2—structure and function in atherosclerosis. *Curr. Opin. Lipidol.* **12**, 121–127.
- Sadovsky, Y. and Crawford, P.A. (1998) Developmental and physiologic roles of the nuclear receptor Steroidogenic factor-1 in the reproductive system. *J. Soc. Gynec. Invest.* **5**, 6–12.
- Saenger, P., Lin, D., Gitelman, S.E. and Miller, W.L. (1993) Congenital lipid adrenal hyperplasia-genes for P450<sub>scc</sub>, side chain cleavage enzyme, are normal. *J. Steroid Biochem. Molec. Biol.* **45**, 87–97.
- Sakai, Y., Yanase, T., Okabe, Y., Hara, T., Waterman, M.R., Takayanagi, R., Haji, M. and Nawata, H. (1994) No mutation in cytochrome P450 side chain cleavage in a patient with congenital lipid adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **79**, 1198–1201.

- Sarria, A.J., Panini, S.R. and Evans, R.M. (1992) A functional role for vimentin intermediate filaments in the metabolism of lipoprotein-derived cholesterol in human SW-13 cells. *J. Biol. Chem.* **267**, 19455–19463.
- Schafer, B.L., Bishop, R.W., Kratunis, V.J., Kalinowski, S.S., Mosley, S.T., Gibson, K.M. and Tanaka, R.D. (1992) Molecular cloning of human mevalonate kinase and identification of a missense mutation in the genetic disease mevalonic aciduria. *J. Biol. Chem.* **267**, 13229–13238.
- Schonfeld, G. (1995) The hypobetalipoproteinemias. *Annu. Rev. Nutr.* **15**, 23–34.
- Schroeder, F., Frolov, A.A., Murphy, E.J., Atshaves, B.P., Jefferson, J.R., Pu, L., Wood, W.G., Foxworth, W.B. and Kier, A.B. (1996) Recent advances in membrane cholesterol domain dynamics and intracellular cholesterol trafficking. *Proc. Soc. Exp. Biol. Med.* **213**, 150–177.
- Schweitzer, S.C. and Evans, R.M. (1998) Vimentin and lipid metabolism. In: *Subcellular Biochemistry, Intermediate Filaments*, Herrman and Harris (eds), Plenum Press, New York, pp. 437–463.
- Servetnick, D.A., Brasaemle, D.L., Gruia-Gray, J., Kimmel, A.R., Wolff, J. and Londos, C. (1995) Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and Leydig cells. *J. Biol. Chem.* **270**, 16970–16973.
- Shackleton, C.H., Roitman, E., Kratz, L.E. and Kelley, R.I. (1999) Equine type estrogens produced by a pregnant woman carrying a Smith-Lemli-Opitz syndrome fetus. *J. Clin. Endocrinol. Metab.* **84**, 1157–1159.
- Shamburek, R.D., Pentchev, P.G., Zech, L.A., Blanchette-Mackie, J., Carstea, E.D., *et al.* (1997) Intracellular trafficking of the free cholesterol derived from LDL cholesteryl ester is defective *in vivo* in Niemann-Pick C disease: insights on normal metabolism of HDL and LDL gained from the NP-C mutation. *J. Lipid Res.* **38**, 2422–2435.
- Sparrow, C.P. and Pittman, R.C. (1990) Cholesterol esters selectively taken up from high-density lipoproteins are hydrolyzed extralysosomally. *Biochim. Biophys. Acta* **1043**, 203–210.
- Stangl, H., Hyatt, M. and Hobbs, H.H. (1999) Transport of lipids from high and low density lipoproteins via scavenger receptor-BI. *J. Biol. Chem.* **274**, 32692–32698.
- Stocco, D.M. (1999) Steroidogenic acute regulatory protein. *Vit. Horm.* **55**, 399–441.
- Stocco, D.M. and Clark, B.J. (1996) Regulation of the acute production of steroids in steroidogenic cells. *Endocr. Rev.* **17**, 221–244.
- Strauss, III, J.F., Kallen, C.B., Christenson, L.K., Watari, H., Devoto, L., Arakane, F., Kiriakidou, M. and Sugawara, T. (1999) The steroidogenic acute regulatory protein (StAR): a window into the complexities of intracellular cholesterol trafficking. *Rec. Prog. Horm. Res.* **54**, 369–395.
- Sugawara, T., Holt, J.A., Driscoll, D., Strauss, III, J.F., Lin, D., *et al.*, (1995a) Human steroidogenic acute regulatory protein: functional activity in COS-1 cells, tissue-specific expression, and mapping of the structural gene to 8p11.2 and a pseudogene to chromosome 13. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4778–4782.
- Sugawara, T., Holt, J.A., Kiriakidou, M. and Strauss, III, J.F. (1996) Steroidogenic factor 1-dependent promoter activity of the human steroidogenic acute regulatory protein (StAR) gene. *Biochemistry* **35**, 9052–9059.
- Sugawara, T., Kiriakidou, M., McAllister, J.M., Kallen, C.B. and Strauss, III, J.F. (1997) Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5′-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. *Biochemistry* **36**, 7249–7255.
- Sugawara, T., Lin, D., Holt, J.A., Martin, K.O., Javitt, N.B., Miller, W.L. and Strauss, III, J.F. (1995b) Structure of the human steroidogenic acute regulatory protein (StAR) gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase activity. *Biochemistry* **34**, 12506–12512.

- Tavani, D.M., Tanaka, T., Strauss, III, J.F. and Billheimer, J.T. (1982) Regulation of acyl coenzyme A: cholesterol acyltransferase in the luteinized rat ovary: observations with an improved enzymatic assay. *Endocrinology* **111**, 794–800.
- Tee, M.K., Lin, D., Sugawara, T., Holt, J.A., Guiguen, Y., Buckingham, B., Strauss, III, J.F. and Miller, W.L. (1995) T→A transversion 11bp from a splice acceptor site in the human gene for steroidogenic acute regulatory protein causes congenital lipid adrenal hyperplasia. *Hum. Mol. Genet.* **4**, 2299–2305.
- Tint, G.S., Irons, M., Elias, E.R., Batta, A.K., Frieden, R., Chen, T.S. and Salen, G. (1994) Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330**, 107–113.
- Tint, G.S., Seller, M., Hughes-Benzie, R., Batta, A.K., Shefer, S., Genest, D., Irons, M., Elias, E. and Salen, G. (1995) Markedly increased tissue concentrations of 7-dehydrocholesterol combined with low levels of cholesterol are characteristic of the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36**, 89–95.
- Tozawa, R., Ishibashi, S., Osuga, J., Yagyu, H., Oka, T., Chen, Z., Ohashi, K., Perrey, S., Shionoiri, F., Yahagi, N., Harada, K., Gotoda, T., Yazaki, Y. and Yamada, N. (1999) Embryonic lethality and defective neural tube closure in mice lacking squalene synthase. *J. Biol. Chem.* **274**, 30843–30848.
- Trigatti, B., Rayburn, H., Vinals, M., Braun, A., Miettinen, H., Penman, M., Hertz, M., Schrenzel, M., Amigo, L., Rigotti, A. and Krieger, M. (1999) Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9322–9327.
- Trzeciak, W.H., Sonnenborn, U., Balkow, C. and Kunau, W.H. (1984) Regulation of steroidogenesis in rat adrenal gland: identification of the bifunctional, hormone-sensitive cholesterol esterase—triacylglycerol lipase enzyme protein and its discrimination from hormone-insensitive lipases. *Mol. Cell. Endocrinol.* **35**, 131–141.
- Uittenbogaard, A., Ying, Y. and Smart, E.J. (1998) Characterization of a cytosolic heat-shock protein-caveolin chaperone complex. Involvement in cholesterol trafficking. *J. Biol. Chem.* **273**, 6525–6532.
- Vanier, M.T., Duthel, S., Rodriguez-Lafrasse, C., Pentchev, P. and Carstea, E.D. (1996) Genetic heterogeneity in Niemann-Pick C disease: a study using somatic cell hybridization and linkage analysis. *Am. J. Hum. Genet.* **58**, 118–125.
- Warnock, D.E., Roberts, C., Lutz, M.S., Blackburn, W.A., Young, Jr., W.W. and Baenziger, J.U. (1993) Determination of plasma membrane lipid mass and composition in cultured Chinese hamster ovary cells using high gradient magnetic affinity chromatography. *J. Biol. Chem.* **268**, 10145–10153.
- Wassif, C.A., Maslen, C., Kachilele-Linjewile, S., Lin, D., Linck, L.M., Connor, W.E., Steiner, R.D. and Porter, F.D. (1998) Mutations in the human sterol  $\Delta^7$ -reductase gene at 11q12–13 cause Smith-Lemli-Opitz syndrome. *Am. J. Hum. Genet.* **63**, 55–62.
- Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C.B., Tomasetto, C., Gerton, G.L., Rio, M.C., Baker, M.E. and Strauss, III, J.F. (1997) MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8462–8467.
- Watari, H., Blanchette-Mackie, E.J., Dwyer, N.K., Click, J.M., Patel, S., Neufeld, E.B., Brady, R.O., Pentchev, P.G. and Strauss, III, J.F. (1999a) Niemann-Pick C1 protein: obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 805–810.

- Watari, H., Blanchette-Mackie, E.J., Dwyer, N.K., Watari, M., Neufeld, E.B., Patel, S., Pentchev, P.G. and Strauss, III, J.F. (1999b) Mutations in the leucine zipper motif and sterol-sensing domain inactivate the Niemann-Pick C1 glycoprotein. *J. Biol. Chem.* **274**, 21861–21866.
- Watari, H., Blanchette-Mackie, G.J., Dwyer, N.K., Sun, G., Click, J.M., Patel, S., Neufeld, E.B., Pentchev, P.G. and Strauss, III, J.F. (2000) NPC1-containing compartment of human granulosa lutein cells: a role in intracellular trafficking of cholesterol supporting steroidogenesis. *Exp. Cell Res.* **255**, 56–66.
- Waterham, H.R., Wijburg, F.A., Hennekam, R.C., Vreken, P., Poll-The, B.T., Dorland, L., Duran, M., Jira, P.E., Smeitink, J.A., Wevers, R.A. and Wanders, R.J. (1998) Smith-Lemli-Opitz syndrome is caused by mutations in the 7-dehydrocholesterol reductase gene. *Am. J. Hum. Genet.* **63**, 329–338.
- Waterman, M.R. (1994) Biochemical diversity of cAMP-dependent transcription of steroid hydroxylase genes in the adrenal cortex. *J. Biol. Chem.* **269**, 27783–27786.
- Wu, J., Kim, J., Li, Q., Kwok, P.Y., Cole, T.G., Cefalu, B., Averna, M. and Schonfeld, G. (1999) Known mutations of apoB account for only a small minority of hypobetalipoproteinemia. *J. Lipid Res.* **40**, 955–959.
- Xie, C., Turley, S.D. and Dietschy, J.M. (1999) Cholesterol accumulation in tissues of the niemann-pick type C mouse is determined by the rate of lipoprotein-cholesterol uptake through the coated-pit pathway in each organ. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11992–11997.
- Yang, X., Iwamoto, K., Wang, M., Artwohl, J., Mason, J.I. and Pang, S. (1993) Inherited congenital adrenal hyperplasia in the rabbit is caused by a deletion in the gene encoding cytochrome P450 cholesterol side-chain cleavage enzyme. *Endocrinology* **132**, 1977–1982.
- Yoo, H.W. and Kim, G.H. (1998) Molecular and clinical characterization of Korean patients with congenital lipoid adrenal hyperplasia. *J. Ped. Endocrinol. Metab.* **11**, 707–711.
- Zazopoulos, E., Lalli, E., Stocco, D.M. and Sassone-Corsi, P. (1997) DNA binding and transcriptional repression by DAX-1 blocks steroidogenesis. *Nature* **390**, 311–315.

## 6. STEROID 21-HYDROXYLASE

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This review will focus on our current knowledge of congenital adrenal hyperplasia (CAH) that is principally caused by a deficiency of steroid 21-hydroxylase. Other deficiencies causing CAH, including 11 $\beta$ -hydroxylase, 17 $\alpha$ -hydroxylase and 3 $\beta$ -hydroxysteroid dehydrogenase, are reviewed elsewhere in this volume.

KEY WORDS: 21-hydroxylase cytochrome P450, CYP21, congenital adrenal hyperplasia, salt-wasting, HLA.

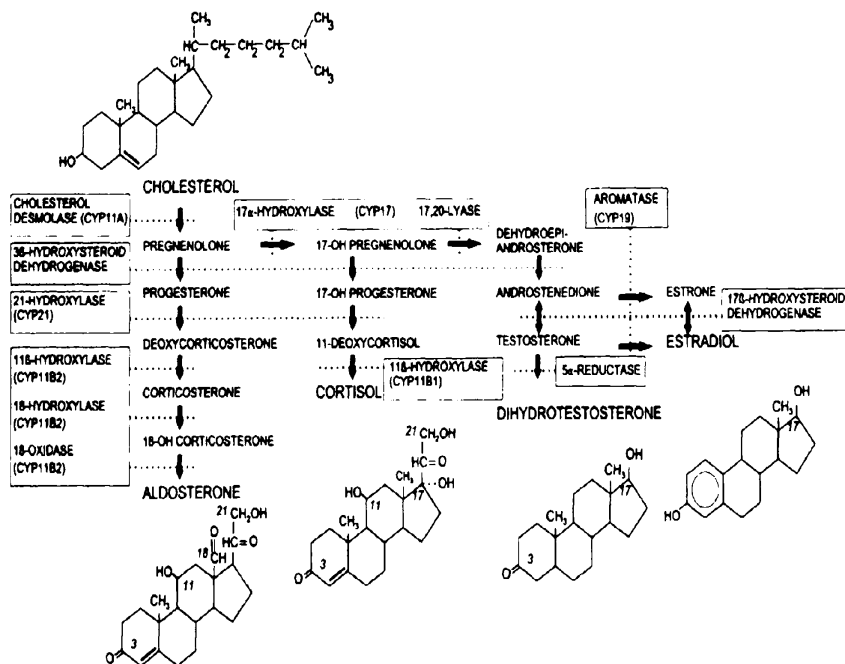
### INTRODUCTION

Cortisol is normally synthesized from cholesterol in the zona fasciculata of the adrenal cortex in five successive enzymatic conversions ([Figure 6.1](#)). Congenital adrenal hyperplasia, the inherited inability to synthesize cortisol, may be caused by defective importation of cholesterol into mitochondria ([Chapter 5](#)) or, more commonly, by mutations in steroidogenic enzymes. More than 90% of cases are caused by a deficiency of the 21-hydroxylase activity required to convert 17-hydroxyprogesterone to 11-deoxycortisol.

### BIOCHEMISTRY OF STEROID 21-HYDROXYLASE

Steroid 21-hydroxylase (CYP21, also known as P450c21) is a microsomal cytochrome P450 enzyme that converts 17-hydroxyprogesterone to 11-deoxycortisol and progesterone to deoxycorticosterone (Kominami *et al.*, 1980, 1984, 1986, 1993; Haniu *et al.*, 1987; Narasimhulu, 1991). Thus, it is required for the biosynthesis of both cortisol and aldosterone. As with other microsomal P450s, the enzyme accepts electrons from an NADPH-dependent cytochrome P450 reductase (Kominami *et al.*, 1984), thus reducing molecular oxygen and hydroxylating the substrate. The P450 reductase is required because NADPH donates electrons in pairs, whereas P450s can only accept single electrons (Sevrioukova and Peterson, 1995).

Human CYP21 normally contains 494 amino acid residues (Higashi *et al.*, 1986; White *et al.*, 1986) (a normal variant has an extra leucine within the N-terminal hydrophobic domain



**Figure 6.1 Pathways of steroid biosynthesis.** The pathways for synthesis of progesterone and mineralocorticoids (aldosterone), glucocorticoids (cortisol), androgens (testosterone and dihydrotestosterone) and estrogens (estradiol) are arranged from left to right. The enzymatic activities catalyzing each bioconversion are written in boxes. For those activities mediated by specific cytochromes P450, the systematic name of the enzyme is listed in parentheses. CYP11B2 and CYP17 have multiple activities. There are several isozymes with 17 $\alpha$ -hydroxysteroid dehydrogenase or 5 $\alpha$ -reductase activity. The planar structures of cholesterol, aldosterone, cortisol, dihydrotestosterone and estradiol are placed near the corresponding labels.

and thus contains 495 residues (Rodrigues *et al.*, 1987)) and has an MW of approximately 52kDa. Human recombinant CYP21 has  $K_m$  values for 17-hydroxyprogesterone and progesterone of 1.2 and 2.8 $\mu$ M respectively, and the apparent  $V_{max}$  for 17-hydroxyprogesterone is twice that of progesterone (Tusie-Luna *et al.*, 1990).

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### Structure-function relationships

Alignment of the amino acid sequences of many P450s have identified a small number of strongly conserved residues which are presumed to be important for catalytic function (Nelson and Strobel, 1989; Black, 1992). The basic three dimensional structure of P450 enzymes has been deduced from X-ray crystallographic studies of two bacterial P450s. The first of these, CYP101 (camphor 5-exo-hydroxylase from *Pseudomonas putida*, also known as P450cam), is a soluble molecule that bears little similarity in primary structure (roughly 15%) to eukaryotic P450s (Poulos, 1991). The second, CYP102 (from *Bacillus megaterium*, also known as P450BM3), is a complex protein consisting of a P450-like N-terminal domain and a C-terminal domain that is 35% identical to eukaryotic cytochrome P450 reductase. The P450 domain is 25–30% identical to the eukaryotic CYP4 and CYP52 families and approximately 20% identical to CYP21. This domain has been subjected to crystallographic analysis (Ravichandran *et al.*, 1993). Its sequence has been aligned with CYP21 and used as the basis for 3-dimensional modeling of CYP21 (Lewis and Lee-Robichaud, 1998). Based on these analyses and functional studies, several conclusions may be drawn.

#### *Heme binding*

The heme iron is critical for catalytic function of P450s. Of its six coordination positions, four interact with the protoporphyrin ring. One of the two axial positions is coordinated to a sulfhydryl group of a completely conserved cysteine (C428 in CYP21), which is located in a relatively highly conserved “heme binding peptide” near the C-terminus. Mutation of C428 in CYP21 destroys enzymatic activity (Wu and Chung, 1991).

#### *Oxygen and water binding*

The ligand at the other axial position of heme is either a water or an oxygen molecule. When an oxygen molecule is bound, it is parallel to the axis of coordination with the iron atom. An H<sub>2</sub>O molecule is consistently present in a groove in the “I” helix adjacent to strongly conserved acidic (aspartate or glutamate) and threonine residues (E294 and T295 in CYP21). According to one of the several proposed models of P450 catalysis, (Rein and Jung, 1993) the first step of the reaction is binding of substrate to oxidized (ferric, Fe<sup>+3</sup>) enzyme. One electron is donated from P450 reductase to the enzyme so that the iron is in the reduced (ferrous, Fe<sup>+2</sup>) state. This complex binds molecular oxygen and then accepts a second electron from the accessory protein, leaving the bound oxygen molecule with a negative charge. Two protons are then donated in succession to the water molecule by the carboxyl group of the acidic residue, transferred to the hydroxyl of the conserved threonine and finally donated to the distal oxygen atom (Ravichandran *et al.*, 1993). The distal oxygen atom is then released as a water molecule, leaving the iron in the Fe<sup>+3</sup> state. The remaining oxygen atom is highly reactive (the iron-oxygen complex is a “ferryl” moiety) and attacks the substrate, resulting in an hydroxylation.



*Substrate binding*

Like most P450 substrates, steroids are relatively hydrophobic molecules. Thus, it is likely that the substrate binding site (s) will consist primarily of hydrophobic amino acid residues.

*A priori*, it was possible that the substrate binding sites of steroid-metabolizing P450s would more closely resemble each other in sequence than the substrate binding sites of other P450s such as xenobiotic metabolizing enzymes. Comparisons of the sequences of 21 and 17-hydroxylase and cholesterol desmolase (CYP21, CYP17 and CYP11A) identified two highly conserved areas, one near the N-terminus (Q53-R60 in CYP21) and the other toward the C-terminus (L342-V358 in CYP21) (White, 1987; Picado-Leonard and Miller, 1988).

The crystal structure of CYP102 confirms that part of the first of these indeed corresponds to a portion of a deep pocket constituting the substrate binding site ( $\beta$ -sheet 1–1, residues E38–A44 in CYP102). However, the second conserved area corresponds to helix K (L311–W325 of CYP102) which does not interact with the substrate. Instead, this region forms part of the docking site for the accessory electron transport protein, cytochrome P450 reductase. The remaining segments that form the substrate binding pocket are widely distributed in the peptide (remainder of  $\beta$ -sheet 1, B' and F helices) and the sequence conservation among steroid metabolizing P450s is not particularly strong in these regions.

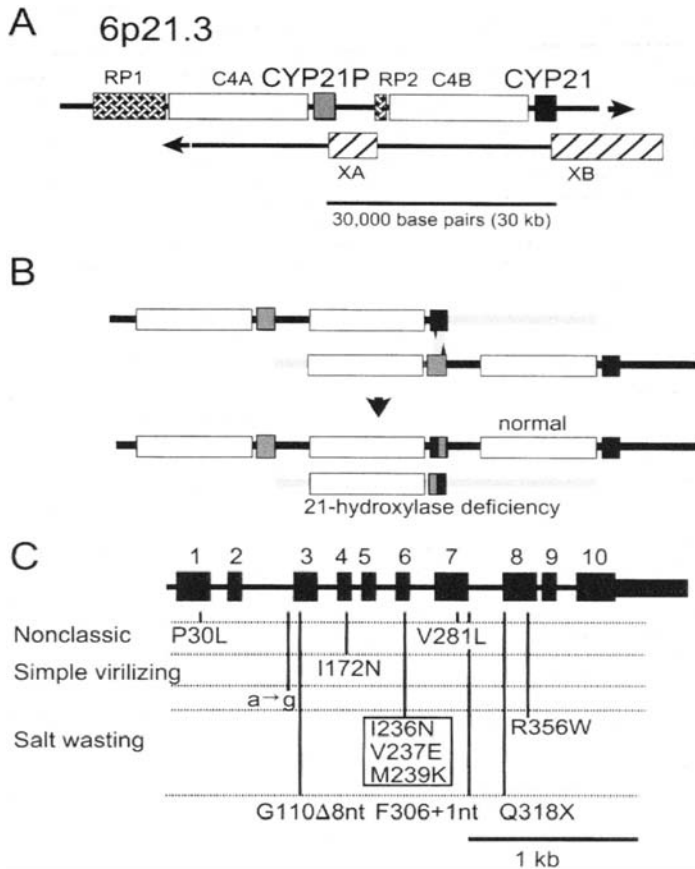
*Binding to accessory proteins*

Microsomal and mitochondrial P450s respectively accept electrons from cytochrome P450 reductase or adrenodoxin. In either case chemical modification studies suggest that basic amino acids (usually lysine) on the P450 interact with acidic residues on the accessory protein. The crystallographic studies of CYP102 suggest a docking site for reductase formed in part by helices B, C, D, J' and K. Helix K, as mentioned, was previously thought to interact with substrate. Support for the idea that it is instead required for redox interactions (with cytochrome P450 reductase or adrenodoxin, depending on the type of P450) comes from mutagenesis studies of CYP11A, wherein modification of either of two lysine residues in helix K destroys enzymatic activity without affecting substrate binding (Wada and Waterman, 1992), and similar studies of CYP17 in which mutagenesis of arginine residues in this region disrupts interactions with cytochrome P450 reductase and cytochrome  $b_5$  (Geller *et al.*, 1999). There are several naturally occurring mutations of arginine residues (R354 and R356) in this region of CYP21 (Lobato *et al.*, 1999; Chiou *et al.*, 1990; Lajic *et al.*, 1997).

Only two basic residues, one in helix K and the other in the heme binding peptide (R323 and R398 in CYP102, corresponding to R354 and R426 in CYP21) are completely conserved in all eukaryotic P450s, suggesting that other positively charged residues that are not completely conserved may be necessary for binding to the accessory protein (Shimizu *et al.*, 1991).

## CYP21 GENE STRUCTURE

The structural gene encoding human CYP21 (CYP21, CYP21A2 or CYP21B) and a pseudogene (CYP21P, CYP21A1P or CYP21A) are located in the *HLA* major



**Figure 6.2 Mutations causing steroid 21-hydroxylase deficiency.** (A) Map of the genetic region around the 21-hydroxylase (CYP21) gene. Arrows denote direction of transcription. CYP21P, 21-hydroxylase pseudogene; C4A and C4B, genes encoding the fourth component of serum complement; RP1, gene encoding a putative nuclear protein of unknown function; RP2, truncated copy of this gene. XB, tenascin-X gene (not shown full length) and XA, a truncated copy of this gene, are on the opposite chromosomal strand. (B) An unequal crossover generating a CYP21 deletion. For clarity, only the two C4 and two CYP21 genes are shown on each chromosome. When these are misaligned during meiosis, a crossover can generate two daughter chromosomes, one with three copies of the C4-CYP21 tandem and the other with one copy. The latter is often a 21-hydroxylase deficiency allele. (C) Diagram of a CYP21P gene. Exons are numbered. CYP21P has nine mutations that may be transferred into CYP21 by gene conversion, causing 21-hydroxylase deficiency. These are arranged in the diagram so that those causing increasing enzymatic compromise are arrayed from top to bottom. Dotted lines divide mutants into groups with similar activities. The mutations at the bottom are nonsense mutations or frameshifts that are predicted to completely prevent protein synthesis. The mutations are associated with different forms of 21-hydroxylase deficiency as marked. As an example of mutation terminology, P30L is Proline-30 to Leucine. a-g, a mutation at nucleotide (nt) 656 in intron 2. The 3 mutations in the box are invariably inherited together. There are several dozen additional rare mutations that collectively account for 5% of all 21-hydroxylase deficiency alleles; these are not shown.—, deletion; +, insertion. Other single letter amino acid codes: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

histocompatibility complex on chromosome 6p21.3 about 30 kilobases (kb) apart, adjacent to and alternating with the C4B and C4A genes encoding the fourth component of serum complement (Carroll *et al.*, 1985a; White *et al.*, 1985) (Figure 6.2). In addition the RP1 (G112) gene is located immediately 5' of C4A and encodes a putative nuclear protein; a truncated copy of this gene, RP2, is located between CYP21P and C4B (Shen *et al.*, 1994). The CYP21, C4 and RP genes are transcribed in the same direction. CYP21 overlaps a gene on the opposite DNA strand (OSG or XB) that encodes a putative extracellular matrix protein similar to tenascin (Bristow *et al.*, 1993b). CYP21P overlaps a truncated copy of this gene (XA) that does not encode a functional protein.

Using pulsed field gel electrophoresis, CYP21 has been mapped approximately 600 kb centromeric of HLA-B and 400 kb telomeric of HLA-DR. It is transcribed in the telomeric to centromeric direction (Dunham *et al.*, 1987; Carroll *et al.*, 1987).

CYP21 and CYP21P each contain 10 exons spaced over 3.1kb. Their nucleotide sequences are 98% identical in exons and about 96% identical in introns (Higashi *et al.*, 1986; White *et al.*, 1986). Mutations in CYP21P are discussed below.

### Other mammalian species

A similar arrangement of genes is found in the mouse except that the genes are located approximately 80kb apart (White *et al.*, 1984a). The positional homolog of CYP21P (i.e., the CYP21 gene on the left if the genes are pictured as being transcribed from left to right) is the active gene in the mouse, whereas the positional homolog of CYP21 is a pseudogene due to a deletion of 215 nucleotides spanning the second exon as well as other nucleotide changes introducing frame shifts and premature termination codons (Chaplin *et al.*, 1986). In addition, the positional homolog of C4A is a pseudogene termed Slp. A mouse model of steroid 21-hydroxylase deficiency (the human form of this disorder is discussed below) is caused by a naturally occurring deletion of the active Cyp21 gene (the active complement C4 gene is also deleted); affected mice die in the neonatal period, presumably from corticosterone and aldosterone deficiency (Gotoh *et al.*, 1988; Shiroishi *et al.*, 1987).

Primates all have two CYP21 genes, one of which is a pseudogene. The mutations inactivating CYP21P all differ, however, from those seen in humans with the exception of the chimpanzee, which does carry an 8bp deletion in exon 3 also seen in humans (Kawaguchi and Klein, 1992; Kawaguchi *et al.*, 1992).

Thus far, a single CYP21 gene has been characterized in cattle (Yoshioka *et al.*, 1986).

## TRANSCRIPTION

### Naturally occurring transcripts

For purposes of this review, the most important gene transcript in the human C4-CYP21 region is that of CYP21 itself, which begins 10–1 Int before the initial AUG codon. Whereas the C4A, C4B and XB genes are mainly expressed in other tissues, the truncated XA gene is transcribed in an adrenal specific manner (Tee *et al.*, 1995b).

CYP21P is also transcribed specifically in the intact adrenal cortex at a level 10–20% that of CYP21 (Bristow *et al.*, 1993a). However, the first two introns are inconsistently spliced out and an uncertain proportion of transcripts include additional exons in the region between the end of CYP21P and the beginning of C4B. Some of these exons may overlap the truncated XA gene (Bristow *et al.*, 1993a). In contrast, CYP21P transcripts cannot be detected in primary cultures of human adrenocortical cells, whereas CYP21 is appropriately expressed under the same conditions (Endoh *et al.*, 1998). In any case, CYP21P transcripts do not contain a long open reading frame and are of uncertain functional significance. Adrenal transcripts in the same direction as CYP21 have also been detected overlapping XB; these are also of uncertain functional significance (Bristow *et al.*, 1993a).

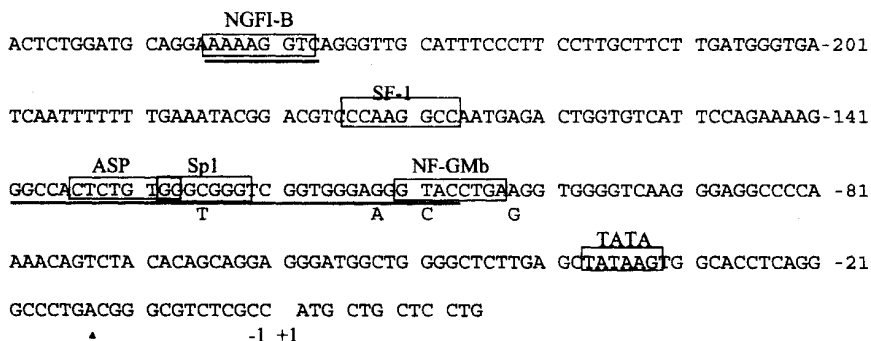
### Hormonally-induced expression

The primary factor regulating CYP21 expression in the zona fasciculata of the adrenal cortex is ACTH (reviewed in Waterman and Bischof, 1997). ACTH induction is mediated mainly through increased transcription (John *et al.*, 1986a, b), is duplicated by cAMP and related agonists such as forskolin, and requires protein kinase A (Handler *et al.*, 1988). This is consistent with the known mode of action of ACTH.

Factors inducing expression of CYP21 in H295R human adrenocortical carcinoma cells, a model of the human zona glomerulosa (the site of aldosterone synthesis), include cAMP (the second messenger for ACTH) and angiotensin II, which acts primarily through the protein kinase C pathway but also through  $\text{Ca}^{2+}$  signaling (Bird *et al.*, 1998). The cAMP and protein kinase C pathways also induce CYP21 expression in primary cultures of human adrenocortical cells, as do insulin and IGF-1 (Endoh *et al.*, 1998).

### 5' Flanking sequences controlling transcription

The 5' flanking region of human CYP21 drives basal expression of reporter constructs at levels 2.5–8 times higher than the corresponding region of CYP21P in cultured mouse Y-1 or human H295 adrenocortical cells (Chang and Chung, 1995; Chin and Chang, 1998); this difference has been localized to the first 176 nucleotides (Chang and Chung, 1995) although sequences upstream of this region are required for full expression (in this discussion, we will number residues from the start of translation, as different numbering systems have been used by different authors). There are only four nucleotide differences between CYP21P and CYP21 in the proximal 176 nucleotides. It appears that the most important differences are at nucleotide –113 which is a G in CYP21 and an A in CYP21P, and at –126, which is a C in CYP21 and a T in CYP21P (Chin and Chang, 1998). The latter polymorphism is in the middle of a binding site for the Spl transcription factor from –123 to –129; the CYP21P sequence binds this factor much less well. Moreover, –126C is at one end of an overlapping binding site for an additional transcription factor termed “adrenal specific protein” (ASP) (Figure 6.3). ASP has not yet been fully characterized but is presumed to bind DNA through “zinc fingers” as do nuclear hormone receptors (Kagawa and Waterman, 1990, 1991, 1992). In contrast, –113G does not lie within a canonical binding site for any known transcription factor, but it is similar to an Spl site and mutation of this nucleotide does interfere with



**Figure 6.3 Transcriptional regulatory sequences in CYP21.** The sequences are numbered from the start of translation so that the +1 position is the first nucleotide of the initial ATG, and the -1 position is the first nucleotide 5' of this. Putative or actual transcription factor binding sites (discussed in the text) (Chang and Chung, 1995) are boxed and marked. Segments known to be required for cAMP regulation (Kagawa and Waterman, 1991) are underlined.

binding of Spl (Chin and Chang, 1998). There is a site at -110/-103 that could bind the nuclear factor NF-GMb, which as far as is known is specific for granulocytes and macrophages (Shannon *et al.*, 1990). Mutating nucleotide-110 does not have major effects on expression, and so the significance of this putative binding site is uncertain.

Another nuclear factor critical for basal expression of mouse Cyp21 (Rice *et al.*, 1990) is steroidogenic factor-1 (SF-1, also called Ad4BP). This is an "orphan" nuclear hormone receptor that is required not only for expression of most steroid hydroxylases but also for the embryonic development of the adrenal gland and gonads (reviewed in Parker and Schimmer, 1997). Human CYP21 contains a consensus SF-1 site (CCAAGGCCA, with the underlined positions being most important for binding) at -169/-175 (White *et al.*, 1986; Higashi *et al.*, 1986). However, to the best of our knowledge the function of the site in the human gene has never been experimentally tested.

Although CYP21 is known to respond to cAMP, the 5' flanking region does not contain a canonical cyclic AMP response element (CRE, TGACGTCA or a variant thereof). Two regions have been implicated in cAMP responsiveness because they confer cAMP responsiveness to heterologous reporter constructs. The first is a segment from -140/-107, which contains the aforementioned Spl and ASP recognition sites (Kagawa and Waterman, 1990, 1991), and the second extends from -244/-237 and binds the transcription factor NGFI-B (nerve growth factor inducible-B, also called Nur77) (Wilson *et al.*, 1993). Mutation of either of these sites destroys cAMP responsiveness (Kagawa and Waterman, 1991; Chang and Chung, 1995); moreover, co-transfection with an NGFI-B expression plasmid transactivates CYP21 reporter constructs (Wilson *et al.*, 1993; Chang and Chung, 1995). NGFI-B is constitutively present in Y-1 mouse adrenocortical cells but is phosphorylated within the DNA binding domain and does not bind DNA. Treatment with ACTH results in *de novo* synthesis of unphosphorylated protein that is able to bind DNA and is transcriptionally active (Li and Lau, 1997).

### More distal elements

Whereas 330 nucleotides of 5' flanking sequences from the mouse CYP21 gene are sufficient for expression of reporter constructs in cultured Y-1 cells (Handler *et al.*, 1988), even 2.2kb of flanking sequences are unable to direct expression to the adrenal gland in transgenic mice (Milstone *et al.*, 1992). At least 6.4kb of such sequences are required; the necessary sequences are localized to two short sequences 5–6kb upstream of CYP21, located within the adjacent Slp gene (an inactive homolog of complement C4; Milstone *et al.*, 1992). These sequences may constitute a locus control region. Such regions are required to establish a tissue-specific open chromatin domain in the vicinity of a particular locus and thus permit appropriate tissue-specific expression. In addition to their effects on chromatin, elements within certain locus control regions can act as transcriptional enhancers, and that is the case for both of the elements in Slp when they are tested in mouse Y-1 cells.

Even these sequences may not be sufficient for full expression of CYP21, because the levels of expression of reporter constructs in transgenic animals are low compared with the intrinsic CYP21 gene (Morley *et al.*, 1996).

It is not yet certain whether a similar region exists in humans, but a cryptic adrenal specific promoter has been located within the C4A gene (Tee *et al.*, 1995a). The finding that the truncated XA gene is expressed specifically in the adrenal gland (Gitelman *et al.*, 1992), although its promoter has been deleted by the duplication of the entire C4-CYP21-X locus, suggests that there is, at least, an adrenal specific enhancer that is able to influence expression of several adjacent genes.

## CLINICAL PRESENTATION OF STEROID 21-HYDROXYLASE DEFICIENCY

Approximately 90–95% of CAH cases are caused by 21-hydroxylase deficiency, which occurs in 1:10,000–1:15,000 births in most populations (Speiser and White, 1998; Wedell, 1998). Patients with 21-hydroxylase deficiency cannot adequately synthesize cortisol because they cannot convert 17-hydroxyprogesterone to 11-deoxycortisol. Many patients also cannot synthesize sufficient aldosterone to maintain sodium balance because they cannot convert progesterone to deoxycorticosterone.

The signs and symptoms of 21-hydroxylase deficiency result both from cortisol and aldosterone deficiencies and from the accumulation of high levels of precursor steroids proximal to the blocked enzymatic step.

### Salt wasting

Approximately three-fourths of patients with the classic form of 21-hydroxylase deficiency are unable to synthesize adequate amounts of aldosterone and are said to have the “salt wasting” form of the disease. Aldosterone is essential for normal sodium homeostasis; deficiency of this hormone results in sodium loss via the kidney, colon and sweat glands (Funder, 1993).

These problems are exacerbated by concomitant cortisol deficiency, which decreases cardiac output and thus glomerular filtration, leading to an inability to excrete free water with consequent hyponatremia. Cortisol deficiency also decreases vascular tone,

exacerbating the hypovolemia caused by aldosterone deficiency. Thus, shock and severe hyponatremia are much more likely in 21-hydroxylase deficiency, in which both cortisol and aldosterone biosynthesis are affected, than in (for example) 11 $\beta$ -hydroxylase or aldosterone synthase deficiencies, in which only one biosynthetic pathway is impaired.

Salt wasting may include such non-specific symptoms as poor appetite, vomiting, lethargy, and failure to gain weight. These patients usually present at 1–4 weeks of age with hyponatremia, hyperkalemia, and hypovolemic shock. Such “salt wasting crises” may prove fatal if not treated.

Elevated plasma renin activity (PRA) is often used as a marker of impaired aldosterone synthesis, but it must be correlated with plasma or urinary aldosterone levels and with sodium balance. Plasma renin can also be elevated in poorly controlled patients with 21-hydroxylase deficiency even if aldosterone synthesis is relatively unimpaired. This occurs because circulating levels of 17-hydroxyprogesterone and progesterone are high, and these steroids may act as antagonists of the mineralocorticoid receptor (Oelkers, 1996).

Although the salt-wasting form of 21-hydroxylase deficiency is genetically determined, siblings may be discordant for salt wasting. Moreover, patients known to have severe salt wasting episodes in infancy and early childhood may show improved sodium balance and more efficient aldosterone synthesis with age (White, 1994). Unrelated but genotypically identical individuals may manifest different severities of salt wasting. Both genetic and non-genetic factors may contribute to the presence or absence of the salt wasting trait. Extra-adrenal 21-hydroxylase has been detected by *in vivo* metabolic studies but the enzyme responsible for this has not been identified.

### Virilization

Because of lack of feedback of cortisol on the hypothalamus and anterior pituitary, ACTH levels are elevated in CAH. Consequently, the adrenal glands become hyperplastic. But rather than cortisol, the adrenals produce excess sex hormone precursors which do not require 21-hydroxylation for their synthesis. Once secreted, these hormones are further metabolized to active androgens (testosterone and dihydrotestosterone) and to a lesser extent estrogens (estrone and estradiol). The net effect is prenatal virilization of girls, and rapid somatic growth with early epiphyseal fusion in both sexes. These problems invariably occur in patients with the salt wasting form of the disease. Patients with adequate aldosterone production and no salt-wasting who nevertheless have signs of prenatal virilization and/or markedly increased production of hormonal precursors of 21-hydroxylase (e.g., 17-hydroxyprogesterone), are termed “simple virilizers”. In addition, a mild “nonclassic” form of the disorder is recognized in which affected females have little or no virilization at birth, but children develop signs of androgen excess during childhood or (for girls) at puberty.

Why do affected females become virilized? Early in gestation, the gonads are indifferent and bipotential. During the 7th week of gestation, the male gonads begin to differentiate under the influence of a cascade of testis-determining genes (Swain *et al.*, 1998). If there is no secretion of anti-Müllerian hormone (AMH), a glycoprotein factor synthesized by the Sertoli cells of the testis (Lee and Donahoe, 1993), development of the Müllerian ducts

proceeds and the female internal structures, the Fallopian tubes, uterus, cervix and upper vagina, are formed. In contrast, development of male genital structures derived from the Wolffian ducts, including the epididymis, ductus deferens, ejaculatory ducts, and seminiferous tubules, requires local high concentrations of testosterone secreted from Leydig cells of the testis beginning at about seven weeks; in the absence of testosterone, Wolffian ducts regress. External genital structures are also bipotential in early gestation, and differentiate as male under the influence of 5 $\alpha$ -dihydrotestosterone (Wilson *et al.*, 1993), which must interact with an intact androgen receptor (Quigley *et al.*, 1995).

Adrenal androgen secretion is increased prenatally in patients with 21-hydroxylase deficiency. This does not significantly affect male sexual differentiation. In affected females, however, the urogenital sinus is in the process of septation when the fetal adrenal begins to produce excess androgens. Blood levels of adrenal androgens are apparently sufficiently high to prevent formation of separate vaginal and urethral canals but not high enough to sustain Wolffian structures. Further interference with normal female genital anatomy occurs as adrenal-derived androgens interact with genital skin androgen receptors and induce clitoral enlargement, promote fusion of the labial folds, and cause rostral migration of the urethral/vaginal perineal orifice. The typical effect in severely affected girls is male appearing genitalia with perineal hypospadias, chordee, and undescended testes. Testosterone exposure *in utero* may also suppress the breast anlage, resulting in poor breast development at adolescence.

Postnatally, adrenal sex steroid production in the untreated or incompletely treated patient with 21-hydroxylase deficiency can cause several problems. Boys have inappropriately rapid somatic growth with accelerated epiphyseal maturation. Pubic hair and apocrine body odor develop, and penile size increases without testicular enlargement. Although early childhood somatic growth is excessive in such patients, adult height is often suboptimal.

Girls may show similar signs of sex steroid excess with progressive clitoral enlargement. In adolescence, poorly controlled girls manifest acne, hirsutism, and ovarian dysfunction. The average age at which menarche occurs is late compared with healthy peers. Poorly treated girls and women often have a clinical picture similar to polycystic ovarian syndrome with sonographic evidence of multiple cysts, anovulation, irregular bleeding and hyperandrogenic symptoms. In general the regularity of menses depends on the adequacy of treatment.

Affected men less frequently manifest impaired gonadal function compared with affected women. Most affected males are able to father children, or at least have normal sperm counts. A prominent complication in incompletely treated males is the development of testicular adrenal rests due to stimulation by ACTH. In severely affected males, testicular tumors accompanied by deficient spermatogenesis may rarely occur despite treatment. These tumors, although most often benign, have prompted biopsies, and sometimes even orchiectomy.

Central precocious puberty may occur when glucocorticoid treatment is optimized in poorly controlled children with markedly advanced bone age. Apparently, chronic exposure to adrenal androgens may cause the hypothalamic-pituitary gonadal axis to "mature." A sudden decrease in androgen levels with adequate treatment may then trigger secretion of



gonadotropins by the pituitary. Clinical suspicion of central precocious puberty in affected boys may be aroused when physical examination in boys reveals increased testicular growth, or if girls show increased breast growth.

## GENETICS OF STEROID 21-HYDROXYLASE DEFICIENCY

### HLA linkage

CAH due to 21-hydroxylase deficiency is inherited as a monogenic autosomal recessive trait closely linked to the HLA complex, meaning that siblings who have 21-hydroxylase deficiency are almost invariably HLA identical (Dupont *et al.*, 1977). In addition, particular forms of 21-hydroxylase deficiency are associated with particular combinations of HLA antigens, or haplotypes; this phenomenon is referred to as genetic linkage disequilibrium. The most interesting is an association between the salt wasting form of the disease and HLA-A3; Bw47; DR7 most characteristically seen in Northern European populations. In addition to 21-hydroxylase deficiency, this haplotype usually carries a null allele at one of the two C4 loci encoding the fourth component of serum complement (O'Neill *et al.*, 1982; Fleischnick *et al.*, 1983). Prior to the cloning of CYP21, this was strongly suspected to represent a contiguous gene syndrome due to a single deletion of C4 and 21 genes; this was confirmed shortly after CYP21 was cloned (White *et al.*, 1984b, 1985). The deletion apparently occurred after the haplotype was generated, because the identical haplotype without the deletion has been identified in the Old Order Amish (Donohoue *et al.*, 1995). The nonclassic form of 21-hydroxylase deficiency is often associated with HLA-B14;DR1, particularly in Eastern European Jewish populations (Kohn *et al.*, 1982; Pollack *et al.*, 1981). This haplotype is associated with the V281L mutation in CYP21 (see below) and with a duplication of complement C4A and the CYP21P pseudogene (Werkmeister *et al.*, 1986; Garlepp *et al.*, 1986). Finally, HLA-A1; B8; DR3 is negatively associated with 21-hydroxylase deficiency. This haplotype has a C4A null allele and is associated with deletion of the C4A and CYP21P genes (White *et al.*, 1985; Carroll *et al.*, 1985b). Thus, comparison of a very few individuals homozygous for HLA-A3; Bw47; DR7 or A1; B8; DR3 strongly suggested that the CYP21 gene (then called the 21-hydroxylase "B" gene) was an active gene, whereas the CYP21P gene (21-hydroxylase "A") was a pseudogene (White *et al.*, 1985).

### Mutations causing 21-hydroxylase deficiency

Most mutations causing 21-hydroxylase deficiency that have been described thus far are apparently the result of either of two types of recombinations between CYP21, the normally active gene, and the CYP21P pseudogene. The two types are unequal crossing-over during meiosis resulting in a complete deletion of C4B and a net deletion of CYP21 (White *et al.*, 1984b; Werkmeister *et al.*, 1986; White *et al.*, 1988), or apparent gene conversion events transferring deleterious mutations normally present in CYP21P to CYP21 (Donohoue *et al.*, 1986b; Harada *et al.*, 1987; Higashi, 1988a; Higashi *et al.*, 1988b; Amor *et al.*, 1988; Globerman *et al.*, 1988; Speiser *et al.*, 1988; Urabe *et al.*, 1990).

The deleterious mutations in CYP21P include an A→G substitution 13 nucleotides (nt) before the end of intron 2 that results in aberrant splicing of pre-mRNA, an 8 nt deletion in exon 3 and a 1 nt insertion in exon 7, each of which shifts the reading frame of translation, and a nonsense mutation in codon 318 of exon 8 (Higashi *et al.*, 1986; White *et al.*, 1986). There are also eight missense mutations in CYP21P, seven of which have been observed in patients with 21-hydroxylase deficiency (Figure 6.2).

Because particular mutations occur in many unrelated kindreds, each mutation, and the degree of enzymatic compromise it causes, may be correlated with the different clinical forms of 21-hydroxylase deficiency (i.e., salt wasting, simple virilizing and nonclassic disease) (Higashi *et al.*, 1991; Mornet *et al.*, 1991; Speiser, 1992a, b; Wedell *et al.*, 1994; Wilson *et al.*, 1995a; Barbat *et al.*, 1995; Ezquieta *et al.*, 1995; Carrera *et al.*, 1996; Levo and Partanen, 1997a; Dardis *et al.*, 1997; Fardella *et al.*, 1998; Ko *et al.*, 1998; Wedell, 1998; Rumsby *et al.*, 1998; Ordóñez-Sánchez *et al.*, 1998)

The functional effects of missense mutations have been assessed *in vitro* by recreating them in CYP21 cDNA and expressing the mutant cDNA using an appropriate expression vector. Several systems have been utilized including transfection of plasmids in mammalian cells (Higashi *et al.*, 1988b; Chiou *et al.*, 1990; Higashi and Fujii-Kuriyama, 1991a; Hsu *et al.*, 1996; Hu *et al.*, 1996; Lajic *et al.*, 1997; Nikoshkov *et al.*, 1997, 1998), infection of mammalian cells with recombinant vaccinia virus (Tusie-Luna *et al.*, 1990, 1991; Helmberg *et al.*, 1992), or expression in yeast (Wu *et al.*, 1991; Wu and Chung, 1991; Hsu *et al.*, 1996; Hu *et al.*, 1996) or bacteria (Hu and Chung, 1990). In general, these systems have yielded similar results regarding the effects of particular mutations on enzymatic activity.

#### *Deletions and large gene conversions*

Large deletions involving C4B and CYP21 comprise approximately 20% of alleles in patients with classic 21-hydroxylase deficiency in most populations. Many deleted alleles are associated with the HLA haplotype A3; Bw47; DR7 (White *et al.*, 1984). Deletions usually extend approximately 30kb from somewhere between exons 3 and 8 of CYP21P through C4B to the corresponding point in CYP21, yielding a single remaining CYP21 gene in which the 5' end corresponds to CYP21P, and the 3' end to CYP21 (White *et al.*, 1988; Donohoue *et al.*, 1989; Gitelman *et al.*, 1992). Deleterious mutations within the CYP21P portion render such a gene incapable of encoding an active enzyme. All patients who carry homozygous deletions suffer from the salt wasting form of the disorder.

One kindred carries an unusual deletion extending into the tenascin-X gene; the patients in this kindred have a contiguous gene syndrome including 21-hydroxylase deficiency and a form of Ehlers-Danlos syndrome due to loss of function of tenascin-X (Burch *et al.*, 1997).

In most studies (White *et al.*, 1984b, 1985; Donohoue *et al.*, 1986a, b; Garlepp *et al.*, 1986; Mornet *et al.*, 1986; Werkmeister *et al.*, 1986; Rumsby *et al.*, 1986; Jospe *et al.*, 1987; Matteson *et al.*, 1987; Dawkins, 1987; White *et al.*, 1988; Rumsby *et al.*, 1988; Morel *et al.*, 1989; Haglund-Stengler *et al.*, 1990, 1991; Mornet *et al.*, 1991; Speiser *et al.*, 1992a, b; Strumberg *et al.*, 1992; Wedell *et al.*, 1994; Ezquieta *et al.*, 1995; Levo and Partanen, 1997a; Lobato *et al.*, 1998), these deletions have been detected by genomic blot hybridization as absence (or diminished intensity in heterozygotes) of gene-specific fragments produced by

digestion with several restriction enzymes. Large gene conversions, in which multiple mutations are transferred from CYP21P to CYP21, are also detected by this approach when gene specific restriction endonuclease sites are affected. Large conversions account for approximately 10% of alleles in classic 21-hydroxylase deficiency.

It is now well appreciated that reliable differentiation of deletions and large gene conversions requires analysis of several different restriction digests (typically using the enzymes *Taq* I and *Bgl* II) in which the sites used to distinguish CYP21P and CYP21 are widely spaced (Matteson *et al.*, 1987; White *et al.*, 1988; Morel *et al.*, 1989). This is required because the remaining CYP21-like gene on a chromosome with a deletion consists of the 3' end of CYP21 "spliced" onto the 5' end of CYP21P, so that the missing fragment in each restriction digest does not necessarily correspond in size to the CYP21-specific fragment in normal chromosomes. In fact, deletions of CYP21 and CYP21P are indistinguishable in certain restriction digests (e.g., *Bgl* II). On the other hand, transfers of polymorphic restriction sites from CYP21P to CYP21 by gene conversion can also be difficult to distinguish from actual deletions of CYP21 in single restriction digests (e.g., *Taq* I).

Moreover, variations in copy number of C4A and CYP21P are extremely frequent in normal individuals. Deletion of C4A and CYP21P is a normal variant present on more than 5% of all chromosomes (White *et al.*, 1985; Carroll *et al.*, 1985a, b), and duplications are also common (Werkmeister *et al.*, 1986). The presence of such a rearrangement on one chromosome can be very confusing when a C4B-CYP21 deletion or a large CYP21 gene conversion is present on the other chromosome. Thus, DNA from both parents should be examined whenever possible to confirm segregation of putative deletions or gene conversions. Reprobing the same blots with a probe for C4 is a useful measure to confirm deletions (Schneider *et al.*, 1986; Rumsby *et al.*, 1986; White *et al.*, 1988).

Deletions have also been directly documented by resolving very large restriction fragments using pulsed field gradient electrophoresis (Collier *et al.*, 1989; Dunham *et al.*, 1989; Zhang *et al.*, 1990) and by high resolution fluorescent *in situ* hybridization (Suto *et al.*, 1996).

In practice, genomic blot hybridizations are no longer routinely used for molecular diagnosis because they are more laborious and less informative than PCR based techniques. However, PCR is unable to distinguish deletions from large gene conversions.

#### *Nonsense and frameshift mutations*

Two other mutations normally found in CYP21P completely prevent synthesis of an intact enzyme and cause salt wasting 21-hydroxylase deficiency if they occur in CYP21: the nonsense mutation in codon 318 (Q318X) (Globerman *et al.*, 1988) and the 8nt deletion in exon 3 (Higashi *et al.*, 1988a). The 1 nt insertion in exon 7 of CYP21P has generally not been identified as an independent mutation in patients with 21-hydroxylase deficiency.

*A or C→G mutation in intron 2*

The nucleotide 13bp before the end of intron 2 (nt656) is A or C in normal individuals. Mutation to G constitutes the single most frequent allele causing classic 21-hydroxylase deficiency.

This mutation causes aberrant splicing of intron 2 with retention of 19 nucleotides normally spliced out of mRNA, resulting in a shift in the translational reading frame (Higashi *et al.*, 1988b, 1991). Almost all of the mRNA is aberrantly spliced, but in cultured cells a small amount of normally spliced mRNA is detected. If no other mutations were present, a small amount of normal enzyme might thus be synthesized.

Although it is not known what proportion of mRNA is normally spliced in the adrenal glands of patients with this mutation, most (but not all) patients who are homozygous or hemizygous for this mutation have the salt-wasting form of the disorder, indicating that they have insufficient enzymatic activity to permit adequate aldosterone synthesis. Occasionally, presentation of salt wasting signs is delayed until several months of age among patients carrying this mutation (Kohn *et al.*, 1995). Putative asymptomatic nt656g homozygotes have been reported but represent PCR typing artifacts (Day *et al.*, 1996).

*Pro-30→Leu (P30L)*

This mutation, which is found in approximately one-sixth of alleles in patients with nonclassic disease, yields an enzyme with 30–50% of normal activity when expressed in cultured cells (Tusie-Luna *et al.*, 1991). Enzymatic activity is, however, rapidly lost when the cells are lysed, suggesting that the enzyme is relatively unstable. Patients carrying this mutation tend to have more severe signs of androgen excess than patients carrying the more common nonclassic mutation V281L (Tusie-Luna *et al.*, 1991; Wedell *et al.*, 1994).

As with other microsomal P450 enzymes, CYP21 is targeted and anchored to the membrane of the endoplasmic reticulum mainly by a hydrophobic “tail” at the amino terminus; this tail is required for enzymatic activity and stability (Hsu *et al.*, 1993). Most P450 enzymes have one or more proline residues separating this tail from the remainder of the polypeptide. These residues are predicted to create a turn in the polypeptide chain, and P30L may abolish this turn. Based on studies in other P450 enzymes, this leads to improper folding of the polypeptide and may interfere with localization in the endoplasmic reticulum (Ishihara *et al.*, 1995). Indeed, the P30L mutant of CYP21 is poorly localized to the endoplasmic reticulum in some studies (Tusie-Luna *et al.*, 1991) but not others (Hu *et al.*, 1996).

*Ile-172→Asn (I172N)*

This mutation, the only one specifically associated with the simple virilizing form of the disease, results in an enzyme with about 1% of normal activity (Tusie-Luna *et al.*, 1990; Chiou *et al.*, 1990) with normal substrate affinity ( $K_m$ ) but reduced activity ( $V_{max}$ ) (Tusie-Luna *et al.*, 1990; Hsu *et al.*, 1996). The isoleucine residue normally at this position in the “E” helix is strongly conserved in many different P450 enzymes and this region of the protein in other P450s interacts with the membrane of the endoplasmic reticulum (Monier

*et al.*, 1988). Mutation of this hydrophobic residue to a polar residue might disrupt such an interaction, weakening the association of the enzyme with the endoplasmic reticulum, and indeed improper localization to the endoplasmic reticulum has been demonstrated in some (Tusie-Luna *et al.*, 1990) but not other (Hsu *et al.*, 1996) studies. Alternatively, the mutation may disrupt an intramolecular hydrophobic interaction stabilizing the secondary structure of the enzyme; the enzyme is abnormally sensitive to protease digestion and does not incorporate heme properly (Hsu *et al.*, 1996).

Because aldosterone is normally secreted at a rate 100–1000 times lower than that of cortisol, it is apparent that 21-hydroxylase activity would have to decrease to very low levels before it became rate-limiting. Apparently, as little as 1% of normal activity allows adequate aldosterone synthesis to prevent significant salt wasting in most cases.

*Ile-Val-Glu-Met-235-238→Asn-Glu-Glu-Lys (I235N/V236E/M238K)*

This cluster of three missense mutations in the “G” helix also abolishes enzymatic activity (Tusie-Luna *et al.*, 1990; Higashi *et al.*, 1991). Interference with substrate binding has been suggested (based on sequence conservation with cholesterol side chain cleavage enzyme, another cytochrome P450) (Higashi *et al.*, 1988b) but is not supported by molecular modeling of CYP21 based on the crystal structure of CYP102.

*Val-281→Leu (V281L)*

V281L occurs in all or nearly all patients with nonclassic 21-hydroxylase deficiency who carry the HLA haplotype B14; DR1, an association that is presumably due to a founder effect (Speiser *et al.*, 1988). In certain populations (such as Jews of Eastern European origin) this is a very common genetic polymorphism with a gene frequency of more than 10% (Speiser *et al.*, 1985; Sherman *et al.*, 1988). Overall, approximately 70% of all nonclassic alleles carry the V281L mutation (Barbat *et al.*, 1995; Blanche *et al.*, 1997). The HLA-B14, DR1 associated haplotype may be less common among nonclassic CAH patients in certain ethnic groups such as Yugoslavs (Dumic *et al.*, 1990). This mutation results in an enzyme with 50% of normal activity when 17-hydroxyprogesterone is the substrate but only 20% of normal activity for progesterone (Tusie-Luna *et al.*, 1990; Wu and Chung, 1991). One study suggested that the mutant enzyme is not normally localized in the endoplasmic reticulum (Tusie-Luna *et al.*, 1990), whereas another proposed that heme binding was affected (Wu and Chung, 1991). As another possibility, this mutation is located in the relatively well conserved “I” helix which contains residues thought to be involved in proton transfer (see above).

*Arg-356→Trp (R356W)*

This mutation abolishes enzymatic activity when expressed in mammalian cells (Chiou *et al.*, 1990; Higashi *et al.*, 1991). It is located in a region of the gene encoding the K helix of the enzyme, which suggests that the mutation affects interactions with the cytochrome P450 reductase, but this has not been demonstrated experimentally (Lajic *et al.*, 1997).

### *Other mutations*

Mutations that are apparently not gene conversions (i.e., they are not usually found in CYP21P) account for 5–10% of 21-hydroxylase deficiency alleles in most populations. The most frequent of these is P453S, which occurs in a number of different populations. This suggests that CYP21P may carry P453S as an occasional polymorphism and that this mutation is transferred to CYP21 in the same way as the other mutations frequently causing 21-hydroxylase deficiency (Helmberg *et al.*, 1992; Owerbach *et al.*, 1992b; Wedell *et al.*, 1992).

Novel mutations are straightforward to detect using automated sequencing technologies in centers with well developed prenatal or neonatal screening programs and have been reported at an increased rate over the past few years. Such mutations include W22X (Lajic and Wedell, 1996), G90V (Lobato *et al.*, 1999), Y97X (Krone *et al.*, 1998), P105L (Wedell *et al.*, 1992; Nikoshkov *et al.*, 1997), G187A (Lobato *et al.*, 1999), deletion of E196 (Nikoshkov *et al.*, 1998), G291S (Wedell, 1992; Nikoshkov *et al.*, 1998), G291C (Lobato *et al.*, 1999), W302X (Levo and Partanen, 1997b), R316X (Lee *et al.*, 1998), R339H (Helmberg *et al.*, 1992), R354H (Lobato *et al.*, 1999), R356P, R356Q (these two are independent of the R356W mutation that can be generated as a gene conversion) (Lajic *et al.*, 1997), E380D (Kirby-Keyser *et al.*, 1997), W405X (Wedell and Luthman, 1993b), R483P (Wedell and Luthman, 1993a) and a frameshift at R483 (Wedell *et al.*, 1992). Larger rearrangements include a deletion of 10 nucleotides in exon 8 and a duplication of 16 nucleotides in exon 9 (Lee *et al.*, 1998). Additional mutations affecting splicing include a mutation of the splice acceptor of intron 1 (Lajic and Wedell, 1996) and the splice donors of introns 2 (Lee *et al.*, 1998) and 7 (Wedell and Luthman, 1993b).

Most of these have been reported on only one chromosome. R339H (Helmberg *et al.*, 1992), R356P, R356Q (Lajic *et al.*, 1997), delE196, G291S and R483P decrease enzymatic activity, and delE196 and R483P also adversely affect enzyme stability (Nikoshkov *et al.*, 1998); P105L acts synergistically with P453S, with which it is associated in one kindred (Nikoshkov *et al.*, 1997).

Despite an apparently exhaustive search, mutations could be not detected in CYP21 in one patient with apparent simple virilizing 21-hydroxylase deficiency (Nimkarn *et al.*, 1999). The patient was homozygous for an HLA haplotype shared (on one chromosome) by a second cousin with salt wasting 21-hydroxylase deficiency, who carried the 8bp deletion in exon 3 on his other chromosome. This strongly suggests that the presumed 21-hydroxylase deficiency in the patient is genetically linked to HLA and thus to CYP21. Trivial explanations aside, this suggests that a site outside the gene is able to significantly influence its expression.

### *Normal polymorphisms*

Several normal polymorphisms have been detected in CYP21 in the course of initial sequencing of cloned genes by several groups (White *et al.*, 1986; Higashi *et al.*, 1986; Rodrigues *et al.*, 1987; Chiou *et al.*, 1990). An extra leucine near the N terminus (this has confused the numbering of other mutations in some reports) and D183E also occur in CYP21P and presumably represent gene conversions that do not affect activity (Higashi *et*

*al.*, 1991). K102R (Rodrigues *et al.*, 1987), S268T (Rodrigues *et al.*, 1987; Donohoue *et al.*, 1990; Wu and Chung, 1991) and N493S (Rodrigues *et al.*, 1987; Chiou *et al.*, 1990) do not represent gene conversions.

### ***De novo* recombinations**

*De novo* mutations of CYP21 must occur frequently because 21-hydroxylase deficiency is often lethal if untreated (thus removing affected alleles from the population) yet it is a relatively common inherited disorder. Indeed, both *de novo* deletions (Sinnott *et al.*, 1990; Hejtmancik *et al.*, 1992) and apparent gene conversions (Speiser *et al.*, 1992a, b; Collier *et al.*, 1993; Wedell *et al.*, 1994) have been documented; the latter usually involve the intron 2 nt656g mutation and comprise approximately 1% of 21-hydroxylase deficiency alleles. In such cases, the proband carries a mutation clearly not inherited from the genetically confirmed parent (s). As the frequency of 21-hydroxylase deficiency alleles in the general population is approximately 2%, the allele frequency of *de novo* gene conversions in intron 2 in the general population should be approximately 1 in  $2 \times 10^4$ .

*De novo* recombinations involving CYP21 have also been documented by PCR in sperm and leukocytes (Tusie-Luna and White, 1995). Unequal crossing-over is detected only in sperm (1 in  $10^5$ – $10^6$  genomes) confirming that this process takes place only during meiosis. Gene conversions, however, take place at equal frequencies in somatic cells and gametes, suggesting that gene conversions occur mainly in mitosis and that meiotic recombination (i.e., double crossing over) contributes little, if at all, to this process. The frequency of gene conversions observed by this strategy (approximately 1 in 104) is consistent with the reported rate of *de novo* gene conversions in patients with 21-hydroxylase deficiency.

The high rate of recombinations involving the CYP21 genes may reflect their location in the major histocompatibility complex, in which a high recombination rate between genes encoding transplantation antigens may increase the diversity of the immune response and be evolutionarily favored. The mechanism by which recombination rates might be increased is not known. It is also not known whether deletions or gene conversions within CYP21 and CYP21P, or more generally within the 30kb tandem duplication containing these genes, take place within certain discrete regions, or "hotspots". It has been suggested that sequences resembling bacteriophage lambda *chi* sites, which are present at relatively high frequencies within CYP21/CYP21P, might promote recombination (Amor *et al.*, 1988), but this hypothesis has not been directly tested.

### **Mutation detection and approaches to prenatal diagnosis**

Genital virilization in affected female fetuses may be minimized by administering dexamethasone to the mother starting as soon as the pregnancy is detected. Dexamethasone crosses the placenta and suppresses fetal ACTH, thus suppressing abnormal production of androgens by the fetal adrenal gland. As this treatment has risks of significant morbidity to the mother, early and accurate prenatal diagnosis permits treatment to be discontinued if the fetus is male or unaffected. This is generally done by genotyping of chorionic villus samples (Mercado *et al.*, 1995; Seckl and Miller, 1997).

Initial attempts to identify mutations in CYP21 gene relied solely on genomic blot hybridization using cDNA probes, which were able to detect only deletions and large gene conversions (i.e., approximately 30% of affected alleles) (Reindollar *et al.*, 1988; Raux-Demay *et al.*, 1989; Speiser *et al.*, 1990; Keller *et al.*, 1991). When it was apparent that other gene conversions accounted for most of the remaining alleles, it became feasible to carry out molecular diagnosis by detection of a limited number of mutations. Gene amplification using the polymerase chain reaction (PCR) dramatically improves the sensitivity of this process. It was, however, initially difficult to use PCR to detect CYP21 mutations because of the paucity of primers that would amplify CYP21 without amplifying the highly homologous CYP21P pseudogene, which already carried most of the mutations of interest. Eventually PCR conditions were identified that permitted gene-specific amplification of CYP21 in two segments. For each of these segments, the CYP21-specific primer includes an 8 base-pair segment in exon 3 that is deleted in CYP21P (Owerbach *et al.*, 1990). This strategy fails to amplify CYP21 if the gene is deleted, but deletions can be detected by conventional Southern blotting. A mutant CYP21 gene would also not be amplified if it contains a gene conversion including exon 3, but such rearrangements can be detected by a second pair of PCRs encompassing exons 1–6 and 6–10. The CYP21-specific primers for these reactions are located in exon 6, in which there is a cluster of 4 nucleotides that is mutated in CYP21P.

Conversely, "back conversion" of CYP21P to include CYP21-specific sequences could lead to spurious amplification of CYP21P and false-positives. This problem is also minimized by amplification of overlapping segments.

PCR-based diagnosis may be complicated by cross-contamination of samples if rigorous controls are not implemented. Furthermore, failure to amplify one haplotype may result in misdiagnosis (Day *et al.*, 1996). Examination of flanking microsatellite markers in all family members can minimize these problems.

Finally, it must be kept in mind that a gene conversion may be sufficiently large that it includes several mutations. If a single DNA sample is amplified, this is impossible to distinguish from compound heterozygosity for different mutations. Therefore, both parents should also be analyzed whenever possible so as to most reliably determine the phase of different mutations (i.e., whether they lie on the same or opposite alleles).

In general, patients are tested for the nine mutations commonly found in CYP21P that can be transferred to CYP21 in gene conversions. As the nine corresponding normal CYP21 sequences must also be tested for, a total of 18 alleles are assayed.

Several approaches have been used to detect these mutations, including allele specific oligonucleotide hybridization (Owerbach *et al.*, 1990, 1992a, b; Mornet *et al.*, 1991; Speiser *et al.*, 1992; Rumsby *et al.*, 1993; Speiser *et al.*, 1994), single stranded conformation polymorphisms (Tajima *et al.*, 1993; Siegel *et al.*, 1994; Bobba *et al.*, 1997; Hayashi *et al.*, 1997; Lee *et al.*, 1998), allele-specific PCR (Wedell and Luthman, 1993b; Wedell *et al.*, 1994; Wilson *et al.*, 1995a, b; Carrera *et al.*, 1997; Wedell, 1998) and ligation detection reactions (Day *et al.*, 1995; Fitness *et al.*, 1999). The best way to make certain that novel mutations are not missed is to sequence the entire gene. This can readily be accomplished using automated sequencing methods (Wedell *et al.*, 1992; Ohlsson and Schwartz, 1997; Krone *et al.*, 1998), and computer analysis eliminates the tedium and potential for error in manually



reading sequencing gels. This technique is most commonly used after other approaches have failed to identify a mutation on at least one known 21-hydroxylase deficiency allele.

### Linked microsatellites

Despite efforts to increase the ease and accuracy of direct mutation detection, it still requires several PCRs. The resulting data are often complex to interpret when gene conversions affect the sites recognized by gene-specific primers. Moreover, as discussed below, certain alleles may be preferentially amplified by PCR, leading to errors in typing. Although this strategy is required for research purposes or for confirmation of neonatal screening results, it may not be necessary or even optimal for prenatal diagnosis, especially in countries lacking a centralized laboratory with expertise in this technique. Linkage analysis using highly polymorphic “microsatellite” loci is an alternative or supplementary technique that can be readily performed by most genetics laboratories (Day *et al.*, 1996).

### Correlations between genotype and phenotype

#### *Classification of disease severity*

As mentioned, patients with 21-hydroxylase deficiency who cannot synthesize aldosterone are termed “salt wasters” and are predisposed to episodic, potentially life-threatening hyponatremic dehydration. Patients with adequate aldosterone production and no salt-wasting who nevertheless have signs of prenatal virilization and/or markedly increased production of hormonal precursors of 21-hydroxylase (e.g., 17-hydroxyprogesterone), are termed “simple virilizers”. In the mild, “nonclassic” form of the disorder, affected females have little or no virilization at birth, but children develop signs of androgen excess during childhood or (for girls) at puberty. This classification into salt wasting, simple virilizing and nonclassic types is a useful way to roughly grade the severity of the disease and to predict the therapeutic interventions that will likely be required. If molecular diagnosis could predict this classification, it would increase the utility of prenatal diagnosis and neonatal screening and it might serve as a useful diagnostic adjunct to ACTH stimulation tests.

The simplest way to correlate genotype and phenotype is to see which mutations are characteristically found in each type of 21-hydroxylase deficiency. This is most informative for frequently occurring mutations. Deletions and large conversions are most often found in salt wasting patients, the intron 2 nt656g mutation is found in both salt wasting and simple virilizing patients, 1172N is characteristically seen in simple virilizing patients, and V281L and P30L are found in nonclassic patients (Mornet *et al.*, 1991; Higzshi *et al.*, 1991; Speiser *et al.*, 1992). This distribution is consistent with the compromise in enzymatic activity conferred by each mutation. However, patients are usually compound heterozygotes for different mutations, and so this approach has little predictive value in itself. A useful analytic strategy is to consider that 21-hydroxylase deficiency is a recessive disease, and thus the phenotype of each patient is likely to reflect his or her less severely impaired allele. If mutations are provisionally classified by the degree of enzymatic compromise—“severe” (also termed “type A”), “moderate” (type B) or “mild” (type C)—then one might

hypothesize that salt wasting patients would have “severe”/“severe” genotypes, simple virilizing patients would have “severe”/“moderate” or “moderate”/“moderate” genotypes, and nonclassic patients would have “severe”/“mild”, “moderate”/“mild” or “mild”/“mild” genotypes. In one study of 88 families (Speiser *et al.*, 1992), these three predictions were correct in 90%, 67% and 59% of cases, respectively. The overall correct classification rate was 79%. An expanded follow-up study of the same population (Wilson *et al.*, 1995a) yielded even better results, with 177/197 patients (88%) being correctly classified in this manner. Similar results were obtained in other studies using the same approach (Wedell *et al.*, 1994; Jaaskelainen *et al.*, 1997).

The salt wasting, simple virilizing and nonclassic categories are qualitative in nature, and the distinction between simple virilizing and nonclassic disease is necessarily difficult in males in whom signs of androgen excess cannot be detected at birth. Therefore, attempts have been made to correlate genotype with quantitative measures of disease severity such as basal and ACTH-stimulated 17-hydroxyprogesterone levels, plasma renin/urinary aldosterone ratios, and Prader genital virilization scores. In general, these are no better correlated with genotype than the broader clinical categories are. There is excellent discrimination between “severe” and “mild” genotypes, but a high degree of overlap between “moderate” genotypes and those either more and less affected (Speiser *et al.*, 1992).

#### *Explanations for “discordance” of genotype and phenotype*

Several explanations for the less than complete correspondence between genotype and phenotype are possible. The most obvious is that the severity of the disease falls on a continuum and patients with disease severity near the “borders” of the various classifications may easily fall on either side of these borders. Several mutations and genotypes seem to be particularly associated with this problem. First, although the intron 2 nt656g mutation is classified as “severe,” it is clearly “leaky” and may yield enough normally spliced mRNA to ameliorate the enzymatic deficiency in some patients. Second, the 1172N mutant has marginal enzymatic function (1% of normal), and this is apparently not always sufficient to prevent salt wasting. These two explanations accounted for 12/20 examples of “discordance” between genotype and phenotype in one study (Wilson *et al.*, 1995a), and significant phenotypic variation was noted in a kindred in which all five affected individuals were compound heterozygotes for these two mutations (Chin *et al.*, 1998). Third, many patients who are “discordant” for genotype and phenotype are compound heterozygotes for mutations that compromise enzymatic activity to different extents (Wilson *et al.*, 1995a; Wedell *et al.*, 1994); thus it appears that some of these patients actually have *in vivo* enzymatic activities intermediate between those seen in patients who are homozygous for each mutation. Consistent with this idea, presumed compound heterozygotes for a classic and nonclassic allele as a group have higher stimulated 17-OHP levels than presumed homozygotes for nonclassic alleles (Speiser and New, 1987).

In studies relying on detection of known mutations, additional novel mutations within CYP21 might adversely affect activity in some cases.

Inaccurate genotyping can obviously confound genotype-phenotype correlations. An important cause of inaccurate genotyping of CYP21 is unequal PCR amplification of different

alleles, sometimes termed "allele dropout". In particular, nt 656g is sometimes preferentially amplified over the corresponding two normal alleles, 656a and especially 656c, so that heterozygous carriers of nt 656g are typed as homozygotes (Day *et al.*, 1996). This led to several reports of high frequencies of asymptomatic homozygotes for this mutation; such individuals were usually obligate heterozygous carriers (such as parents of patients) detected in family studies (Schulze *et al.*, 1995; Witchel *et al.*, 1996; Rumsby *et al.*, 1996).

Finally, genetic or environmental factors other than 21-hydroxylase activity may influence phenotype. The degree of salt wasting tends to improve with time, even in subjects who are genetically predicted to have no 21-hydroxylase activity (Speiser *et al.*, 1991), and genetically identical siblings are occasionally discordant for severity of salt wasting (Stoner *et al.*, 1986; Speiser *et al.*, 1992). Thus this clinical parameter must be influenced by other factors. Similarly, genetically based variations in androgen biosynthesis or sensitivity to androgens would be expected to influence the phenotype (Carrera *et al.*, 1996).

### SUMMARY

Over 90% of cases of virilizing congenital adrenal hyperplasia (CAH, the inherited inability to synthesize cortisol) are caused by 21-hydroxylase deficiency. Females with severe, classic 21-hydroxylase deficiency are exposed to excess androgens prenatally and are born with virilized external genitalia. About three-quarters of patients cannot synthesize sufficient aldosterone to maintain sodium balance and may develop potentially fatal "salt wasting" crises if not treated. In the mild "nonclassic" form of the disorder, affected females have little or no virilization at birth but either sex may develop signs of androgen excess postnatally. The disease is caused by mutations in the CYP21 gene encoding the steroid 21-hydroxylase enzyme. Over 90% of these mutations result from intergenic recombinations between CYP21 and the closely linked CYP21P pseudogene. Approximately 20% of these are gene deletions due to unequal crossing over during meiosis, whereas the remainder represent transfers to CYP21 of deleterious mutations normally present in CYP21P, a process termed gene conversion that apparently takes place during mitosis. The degree to which each mutation compromises enzymatic activity is strongly but not completely correlated with the clinical severity of the disease in patients carrying it. Prenatal diagnosis by direct mutation detection permits prenatal treatment of affected females to minimize genital virilization. Neonatal screening by hormonal methods identifies affected children before salt wasting crises develop, reducing mortality from this condition. Glucocorticoid and mineralocorticoid replacement are the mainstays of treatment, but more rational dosing and additional therapies are being developed.

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# REFERENCES

- Amor, M., Parker, K.L., Globberman, H., New, M.I. and White, P.C. (1988) Mutation in the CYP21B gene (Ile-172→Asn) causes steroid 21-hydroxylase deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1600–1604.
- Barbat, B., Bogyo, A., Raux-Demay, M.C., Kuttann, F., Boue, J., Simon-Bouy, B., Serre, J.L. and Mornet, E. (1995) Screening of CYP21 gene mutations in 129 French patients affected by steroid 21-hydroxylase deficiency. *Hum. Mut.* **5**, 126–130.
- Bird, I.M., Mason, J.I. and Rainey, W.E. (1998) Protein kinase A, protein kinase C, and Ca(2+)-regulated expression of 21-hydroxylase cytochrome P450 in H295R human adrenocortical cells. *J. Clin. Endocrinol. Metab.* **83**, 1592–1597.
- Black, S.D. (1992) Membrane topology of the mammalian P450 cytochromes. *FASEB J.* **6**, 680–685.
- Blanche, H., Vexiau, P., Clauin, S., Le Gall, I., Fiet, J., Mornet, E., Dausset, J. and Bellanne-Chantelot, C. (1997) Exhaustive screening of the 21-hydroxylase gene in a population of hyperandrogenic women. *Hum. Genet.* **101**, 56–60.
- Bobba, A., Iolascon, A., Giannattasio, S., Albrizio, M., Sinisi, A., Prisco, F., Schettini, F. and Marra, E. (1997) Characterisation of CAH alleles with non-radioactive DNA single strand conformation polymorphism analysis of the CYP21 gene. *J. Med. Genet.* **34**, 223–228.
- Bristow, J., Gitelman, S.E., Tee, M.K., Staels, B. and Miller, W.L. (1993a) Abundant adrenal-specific transcription of the human P450c21A “pseudogene”. *J. Biol. Chem.* **268**, 12919–12924.
- Bristow, J., Tee, M.K., Gitelman, S.E., Mellon, S.H. and Miller, W.L. (1993b) Tenascin-X: a novel extracellular matrix protein encoded by the human XB gene overlapping P450c21B. *J. Cell Biol.* **122**, 265–278.
- Burch, G.H., Gong, Y., Liu, W., Dettman, R.W., Curry, C.J., Smith, L., Miller, W.L. and Bristow, J. (1997) Tenascin-X deficiency is associated with Ehlers-Danlos syndrome [see comments]. *Nat. Genet.* **17**, 104–108.
- Carrera, P., Barbieri, A.M., Ferrari, M., Righetti, P.G., Perego, M. and Gelfi, C. (1997) Rapid detection of 21-hydroxylase deficiency mutations by allele-specific *in vitro* amplification and capillary zone electrophoresis. *Clin. Chem.* **43**, 2121–2127.
- Carrera, P., Bordone, L., Azzani, T., Brunelli, V., Garancini, M.P., Chiumello, G. and Ferrari, M. (1996) Point mutations in Italian patients with classic, non-classic, and cryptic forms of steroid 21-hydroxylase deficiency. *Hum. Genet.* **98**, 662–665.
- Carroll, M.C., Campbell, R.D. and Porter, R.R. (1985a) Mapping of steroid 21-hydroxylase genes adjacent to complement component C4 genes in HLA, the major histocompatibility complex in man. *Proc. Natl Acad. Sci. U.S.A.* **82**, 521–525.
- Carroll, M.C., Katzman, P., Alicot, E.M., Roller, B.H., Geraghty, D.E., Orr, H.T., Strominger, J.L. and Spies, T. (1987) Linkage map of the human major histocompatibility complex including the tumor necrosis factor genes. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8535–8539.
- Carroll, M.C., Palsdottir, A., Belt, K.T. and Porter, R.R. (1985b) Deletion of complement C4 and steroid 21-hydroxylase genes in the HLA class III region. *EMBO J.* **4**, 2547–2552.
- Chang, S.F. and Chung, B.C. (1995) Difference in transcriptional activity of two homologous CYP21A genes. *Mol. Endocrinol.* **9**, 1330–1336.
- Chaplin, D.D., Galbraith, L.J., Seidman, J.G., White, P.C. and Parker, K.L. (1986) Nucleotide sequence analysis of murine 21-hydroxylase genes: mutations affecting gene expression. *Proc. Natl Acad. Sci. U.S.A.* **83**, 9601–9605.

- Chin, D., Speiser, P.W., Imperato-McGinley, J., Dixit, N., Uli, N., David, R. and Oberfield, S.E. (1998) Study of a kindred with classic congenital adrenal hyperplasia: diagnostic challenge due to phenotypic variance. *J. Clin. Endocrinol. Metab.* **83**, 1940–1945.
- Chin, K.K. and Chang, S.F. (1998) The -104G nucleotide of the human CYP21 gene is important for CYP21 transcription activity and protein interaction. *Nucl. Acids Res.* **26**, 1959–1964.
- Chiou, S.H., Hu, M.C. and Chung, B.C. (1990) A missense mutation at Ile172Asn or Arg356Trp causes steroid 21-hydroxylase deficiency. *J. Biol. Chem.* **265**, 3549–3552.
- Chung, B.C., Matteson, K.J. and Miller, W.L. (1986) Structure of a bovine gene for P-450c21 (steroid 21-hydroxylase) defines a novel cytochrome P-450 gene family. *Proc. Nat. Acad. Sci. USA.* **83**, 4243–4247.
- Collier, S., Sinnott, P.J., Dyer, P.A., Reincke, M., Harris, R. and Strachan, T. (1989) Pulsed field gel electrophoresis identifies a high degree of variability in the number of tandem 21-hydroxylase and complement C4 gene repeats in 21-hydroxylase deficiency haplotypes. *EMBO J.* **8**, 1393–1402.
- Collier, S., Tassabehji, M. and Strachan, T. (1993) A *de novo* pathological point mutation at the 21-hydroxylase locus: implications for gene conversion in the human genome. *Nat. Genet.* **3**, 260–265.
- Dardis, A., Bergada, I., Bergada, C., Rivarola, M. and Belgorosky, A. (1997) Mutations of the steroid 21-hydroxylase gene in an Argentinian population of 36 patients with classical congenital adrenal hyperplasia. *J. Ped. Endocrinol. Metabol.* **10**, 55–61.
- Dawkins, R.L., Martin, E., Kay, P.H., Garlepp, M.J., Wilton, A.N. and Stuckey, M.S. (1987) Heterogeneity of steroid 21-hydroxylase genes in classical congenital adrenal hyperplasia. *J. Immunogenet.* **14**, 89–98.
- Day, D.J., Speiser, P.W., Schultz, E., Bettendorf, M., Fitness, J., Barany, F. and White, P.C. (1996) Identification of non-amplifying CYP21 genes when using PCR-based diagnosis of 21-hydroxylase deficiency in congenital adrenal hyperplasia (CAH) affected pedigrees. *Hum. Mol. Genet.* **5**, 2039–2048.
- Day, D.J., Speiser, P.W., White, P.C. and Barany, F. (1995) Detection of steroid 21-hydroxylase alleles using gene-specific PCR and a multiplexed ligation detection reaction. *Genomics* **29**, 152–162.
- Donohoue, P.A., Guethlein, L., Collins, M.M., Van Dop, C., Migeon, C.J., Bias, W.B. and Schmeckpeper, B.J. (1995) The HLA-A3, Cw6, B47, DR7 extended haplotypes in salt losing 21-hydroxylase deficiency and in the Old Order Amish: identical class I antigens and class II alleles with at least two crossover sites in the class III region. *Tissue Antigens* **46**, 163–172.
- Donohoue, P.A., Jospe, N., Migeon, C.J., McLean, R.H., Bias, W.B., White, P.C. and Van Dop, C. (1986a) Restriction maps and restriction fragment length polymorphisms of the human 21-hydroxylase genes [published erratum appears in *Biochem. Biophys. Res. Commun.* **138**, 503, 1986]. *Biochem. Biophys. Res. Commun.* **136**, 722–729.
- Donohoue, P.A., Jospe, N., Migeon, C.J. and Van Dop, C. (1989) Two distinct areas of unequal crossingover within the steroid 21-hydroxylase genes produce absence of CYP21B [published erratum appears in *Genomics* **6**, 392, 1990]. *Genomics* **5**, 397–406.
- Donohoue, P.A., Sandrini Neto, R., Collins, M.M. and Migeon, C.J. (1990) Exon 7 NcoI restriction site within CYP21B (steroid 21-hydroxylase) is a normal polymorphism. *Mol. Endocrinol.* **4**, 1354–1362.
- Donohoue, P.A., Van Dop, C., McLean, R.H., White, P.C., Jospe, N. and Migeon, C.J. (1986b) Gene conversion in salt-losing congenital adrenal hyperplasia with absent complement C4B protein. *J. Clin. Endocrinol. Metab.* **62**, 995–1002.

- Dumic, M., Brkljacic, L., Speiser, P.W., Wood, E., Crawford, C., Plavsic, V., Baniceviac, M., Radmanovic, S., Radica, A., Kastelan, A. *et al.*, (1990) An update on the frequency of nonclassic deficiency of adrenal 21-hydroxylase in the Yugoslav population. *Acta Endocrinol. (Copenh)* **122**, 703–710.
- Dunham, L, Sargent, C.A., Dawkins, R.L. and Campbell, R.D. (1989) Direct observation of the gene organization of the complement C4 and 21-hydroxylase loci by pulsed field gel electrophoresis. *J. Exp. Med.* **169**, 1803–1818.
- Dunham, L, Sargent, C.A., Trowsdale, J. and Campbell, R.D. (1987) Molecular mapping of the human major histocompatibility complex by pulsed-field gel electrophoresis. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7237–7241.
- Dupont, B., Oberfield, S.E., Smithwick, E.M., Lee, T.D. and Levine, L.S. (1977) Close genetic linkage between HLA and congenital adrenal hyperplasia (21-hydroxylase deficiency). *Lancet* **ii**, 1309–1312.
- Endoh, A., Yang, L. and Hornsby, P.J. (1998) CYP21 pseudogene transcripts are much less abundant than those from the active gene in normal human adrenocortical cells under various conditions in culture. *Mol. Cell. Endocrinol.* **137**, 13–19.
- Ezquieta, B., Oliver, A., Gracia, R. and Gancedo, P.G. (1995) Analysis of steroid 21-hydroxylase gene mutations in the Spanish population. *Hum. Genet.* **96**, 198–204.
- Fardella, C.E., Poggi, H., Pineda, P., Soto, J., Torrealba, I., Cattani, A, Oestreicher, E. and Foradori, A. (1998) Salt-wasting congenital adrenal hyperplasia: detection of mutations in CYP21B gene in a Chilean population. *J. Clin. Endocrinol. Metab.* **83**, 3357–3360.
- Fitness, J., Dixit, N., Webster, D., Torresani, T., Pergolizzi, R., Speiser, P.W. and Day, D.J. (1999) Genotyping of CYP21, linked chromosome 6p markers, and a sex-specific gene in neonatal screening for congenital adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **84**, 960–966.
- Fleischnick, E., Awdeh, Z.L., Raum, D., Granados, J., Alosco, S.M., Crigler, J.F.J., Gerald, P.S., Giles, C.M., Yunis, E.J. and Alper, C.A. (1983) Extended MHC haplotypes in 21-hydroxylase-deficiency congenital adrenal hyperplasia: shared genotypes in unrelated patients. *Lancet* **i**, 152–156.
- Funder, J.W. (1993) Aldosterone action. *Annu. Rev. Physiol.* **55**, 115–130.
- Garlepp, M.J., Wilton, AN., Dawkins, R.L. and White, P.C. (1986) Rearrangement of 21-hydroxylase genes in disease-associated MHC supratypes. *Immunogenetics* **23**, 100–105.
- Geller, D.H., Auchus, R.J. and Miller, W.L. (1999) P450c17 mutations R347H and R358Q selectively disrupt 17,20-lyase activity by disrupting interactions with P450 oxidoreductase and cytochrome b<sub>5</sub>. *Mol. Endocrinol.* **13**, 167–175.
- Gitelman, S.E., Bristow, J. and Miller, W.L. (1992) Mechanism and consequences of the duplication of the human C4/P450c2 1/gene X locus [published erratum appears in Mol. Cell Biol. 1992 Jul; 12 (7):3313–4]. *Mol. Cell. Biol.* **12**, 2124–2134.
- Globerman, H., Amor, M., Parker, K.L., New, M.I. and White, P.C. (1988) Nonsense mutation causing steroid 21-hydroxylase deficiency. *J. Clin. Invest.* **82**, 139–144.
- Gotoh, H., Sagai, T., Hata, J., Shiroishi, T. and Moriwaki, K. (1988) Steroid 21-hydroxylase deficiency in mice. *Endocrinology* **123**, 1923–1927.
- Haglund-Stengler, B., Ritzén, E.M., Gustafsson, J. and Luthman, H. (1991) Haplotypes of the steroid 21-hydroxylase gene region encoding mild steroid 21-hydroxylase deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8352–8356.
- Haglund-Stengler, B., Ritzén, E.M. and Luthman, H. (1990) 21-hydroxylase deficiency: disease-causing mutations categorized by densitometry of 21-hydroxylase-specific deoxyribonucleic acid fragments. *J. Clin. Endocrinol. Metab.* **70**, 43–48.

- Handler, J.D., Schimmer, B.P., Flynn, T.G., Szyf, M., Seidman, J.G. and Parker, K.L. (1988) An enhancer element and a functional cyclic AMP-dependent protein kinase are required for expression of adrenocortical 21-hydroxylase. *J. Biol. Chem.* **263**, 13068–13073.
- Haniu, M., Yanagibashi, K., Hall, P.F. and Shively, J.E. (1987) Complete amino acid sequence of 21-hydroxylase cytochrome P-450 from porcine adrenal microsomes. *Arch. Biochem. Biophys.* **254**, 380–384.
- Harada, F., Kimura, A., Iwanaga, T., Shimozaawa, K., Yata, J. and Sasazuki, T. (1987) Gene conversion-like events cause steroid 21-hydroxylase deficiency in congenital hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8091–8094.
- Hayashi, Z., Orimo, H., Araki, T. and Shimada, T. (1997) Prenatal diagnosis of steroid 21-hydroxylase deficiency by analysis of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) profiles. *Prenat. Diagn.* **17**, 435–442.
- Hejtmancik, J.F., Black, S., Harris, S., Ward, P.A., Callaway, C., Ledbetter, D., Morris, J., Leech, S.H. and Pollack, M.S. (1992) Congenital 21-hydroxylase deficiency as a new deletion mutation. Detection in a proband during subsequent prenatal diagnosis by HLA typing and DNA analysis. *Hum. Immunol.* **35**, 246–252.
- Helmberg, A., Tusie-Luna, M.T., Tabarelli, M., Kofler, R. and White, P.C. (1992) R339H and P453S: CYP21 mutations associated with nonclassic steroid 21-hydroxylase deficiency that are not apparent gene conversions. *Mol. Endocrinol.* **6**, 1318–1322.
- Higashi, Y. and Fujii-Kuriyama, Y. (1991) Functional analysis of mutant P450 (C21) genes in COS cell expression system. *Methods Enzymol.* **206**, 166–173.
- Higashi, Y., Hiromasa, T., Tanae, A., Miki, T., Nakura, J., Kondo, T., Ohura, T., Ogawa, E., Nakayama, K. and Fujii-Kuriyama, Y. (1991) Effects of individual mutations in the P-450 (C21) pseudogene on the P-450 (C21) activity and their distribution in the patient genomes of congenital steroid 21-hydroxylase deficiency. *J. Biochem. (Tokyo)* **109**, 638–644.
- Higashi, Y., Tanae, A., Inoue, H. and Fujii-Kuriyama, Y. (1988a) Evidence for frequent gene conversion in the steroid 21-hydroxylase P-450 (C21) gene: implications for steroid 21-hydroxylase deficiency. *Am. J. Hum. Genet.* **42**, 17–25.
- Higashi, Y., Tanae, A., Inoue, H., Hiromasa, T. and Fujii-Kuriyama, Y. (1988b) Aberrant splicing and missense mutations cause steroid 21-hydroxylase [P-450 (C21)] deficiency in humans: possible gene conversion products. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7486–7490.
- Higashi, Y., Yoshioka, H., Yamane, M., Gotoh, O. and Fujii-Kuriyama, Y. (1986) Complete nucleotide sequence of two steroid 21-hydroxylase genes tandemly arranged in human chromosome: a pseudogene and a genuine gene. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2841–2845.
- Hsu, L.C., Hsu, N.C., Guzova, J.A., Guzov, V.M., Chang, S.F. and Chung, B.C. (1996) The common 1172N mutation causes conformational change of cytochrome P450c21 revealed by systematic mutation, kinetic, and structural studies. *J. Biol. Chem.* **271**, 3306–3310.
- Hsu, L.C., Hu, M.C., Cheng, H.C., Lu, J.C. and Chung, B.C. (1993) The N-terminal hydrophobic domain of P450c21 is required for membrane insertion and enzyme stability. *J. Biol. Chem.* **268**, 14682–14686.
- Hu, M.C. and Chung, B.C. (1990) Expression of human 21-hydroxylase (P450c21) in bacterial and mammalian cells: a system to characterize normal and mutant enzymes. *Mol. Endocrinol.* **4**, 893–898.
- Hu, M.C., Hsu, L.C., Hsu, N.C. and Chung, B.C. (1996) Function and membrane topology of wild-type and mutated cytochrome P-450c21. *Biochem. J.* **316**, 325–329.
- Ishihara, N., Yamashina, S., Sakaguchi, M., Mihara, K. and Omura, T. (1995) Malfolded cytochrome P-450 (M1) localized in unusual membrane structures of the endoplasmic reticulum in cultured animal cells. *J. Biochem. (Tokyo)* **118**, 397–404.

- Jaaskelainen, J., Levo, A., Voutilainen, R. and Partanen, J. (1997) Population-wide evaluation of disease manifestation in relation to molecular genotype in steroid 21-hydroxylase (CYP21) deficiency: good correlation in a well defined population. *J. Clin. Endocrinol. Metab.* **82**, 3293–3297.
- John, M.E., John, M.C., Boggaram, V., Simpson, E.R. and Waterman, M.R. (1986a) Transcriptional regulation of steroid hydroxylase genes by corticotropin. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4715–4719.
- John, M.E., Okamura, T., Dee, A., Adler, B., John, M.C., White, P.C., Simpson, E.R. and Waterman, M.R. (1986b) Bovine steroid 21-hydroxylase: regulation of biosynthesis. *Biochemistry* **25**, 2846–2853.
- Jospe, N., Donohoue, P.A., Van Dop, C., McLean, R.H., Bias, W.B. and Migeon, C.J. (1987) Prevalence of polymorphic 21-hydroxylase gene (CA21HB) mutations in salt-losing congenital adrenal hyperplasia. *Biochem. Biophys. Res. Commun.* **142**, 7987–804.
- Kagawa, N. and Waterman, M.R. (1990) cAMP-dependent transcription of the human CYP21B (P-450C21) gene requires a cis-regulatory element distinct from the consensus cAMP-regulatory element. *J. Biol. Chem.* **265**, 11299–11305.
- Kagawa, N. and Waterman, M.R. (1991) Evidence that an adrenal-specific nuclear protein regulates the cAMP responsiveness of the human CYP21B (P450C21) gene. *J. Biol. Chem.* **266**, 11199–11204.
- Kagawa, N. and Waterman, M.R. (1992) Purification and characterization of a transcription factor which appears to regulate cAMP responsiveness of the human CYP21B gene. *J. Biol. Chem.* **267**, 25213–25219.
- Kawaguchi, H. and Klein, J. (1992) Organization of C4 and CYP21 loci in gorilla and orangutan. *Hum. Immunol.* **33**, 153–162.
- Kawaguchi, H., O'h Uigin, C. and Klein, J. (1992) Evolutionary origin of mutations in the primate cytochrome P450c21 gene. *Am. J. Hum. Genet.* **50**, 766–780.
- Keller, E., Andreas, A., Scholz, S., Dorr, H.C., Knorr, D. and Albert, E.D. (1991) Prenatal diagnosis of 21-hydroxylase deficiency by RFLP analysis of the 21-hydroxylase, complement C4, and HLA class II genes. *Prenat. Diagn.* **11**, 827–840.
- Kirby-Keyser, L., Porter, C.C. and Donohoue, P.A. (1997) E380D: a novel point mutation of CYP21 in an HLA-homozygous patient with salt-losing congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Human Mutation* **9**, 181–182.
- Ko, T.M., Kao, C.H., Ho, H.N., Tseng, L.H., Hwa, H.L., Hsu, P.M., Chuang, S.M. and Lee, T.Y. (1998) Congenital adrenal hyperplasia. Molecular characterization. *J. Reprod. Med.* **43**, 379–386.
- Kohn, B., Day, D., Alemzadeh, R., Enerio, D., Patel, S.V., Pelczar, J.V. and Speiser, P.W. (1995) Splicing mutation in CYP21 associated with delayed presentation of salt-wasting congenital adrenal hyperplasia. *Am. J. Med. Genet.* **57**, 450–454.
- Kohn, B., Levine, L.S., Pollack, M.S., Pang, S., Lorenzen, F., Levy, D., Lerner, A.J., Rondonini, G.F., Dupont, B. and New, M.I. (1982) Late-onset steroid 21-hydroxylase deficiency: a variant of classical congenital adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **55**, 817–827.
- Kominami, S., Hara, H., Ogishima, T. and Takemori, S. (1984) Interaction between cytochrome P-450 (P-450C21) and NADPH-cytochrome P-450 reductase from adrenocortical microsomes in a reconstituted system. *J. Biol. Chem.* **259**, 2991–2999.
- Kominami, S., Itoh, Y. and Takemori, S. (1986) Studies on the interaction of steroid substrates with adrenal microsomal cytochrome P-450 (P-450C21) in liposome membranes. *J. Biol. Chem.* **261**, 2077–2083.



- Kominami, S., Ochi, H., Kobayashi, Y. and Takemori, S. (1980) Studies on the steroid hydroxylation system in adrenal cortex microsomes. Purification and characterization of cytochrome P-450 specific for steroid C-21 hydroxylation. *J. Biol. Chem.* **255**, 3386–3394.
- Kominami, S., Tagashira, H., Ohta, Y., Yamada, M., Kawato, S. and Takemori, S. (1993) Membrane topology of bovine adrenocortical cytochrome P-450C21: structural studies by trypsin digestion in vesicle membranes. *Biochemistry* **32**, 12935–12940.
- Krone, N., Roscher, A.A., Schwarz, H.P. and Braun, A. (1998) Comprehensive analytical strategy for mutation screening in 21-hydroxylase deficiency. *Clin. Chem.* **44**, 2075–2082.
- Lajic, S., Levo, A., Nikoshkov, A., Lundberg, Y., Partanen, J. and Wedell, A. (1997) A cluster of missense mutations at Arg356 of human steroid 21-hydroxylase may impair redox partner interaction. *Hum. Genet.* **99**, 704–709.
- Lajic, S. and Wedell, A. (1996) An intron 1 splice mutation and a nonsense mutation (W23X) in CYP21 causing severe congenital adrenal hyperplasia. *Hum. Genet.* **98**, 182–184.
- Lee, H.H., Chao, H.T., Lee, Y.J., Shu, S.G., Chao, M.C., Kuo, J.M. and Chung, B.C. (1998) Identification of four novel mutations in the CYP21 gene in congenital adrenal hyperplasia in the Chinese. *Hum. Genet.* **103**, 304–310.
- Lee, M.M. and Donahoe, P.K. (1993) Mullerian inhibiting substance: a gonadal hormone with multiple functions. [Review] *Endocr. Rev.* **14**, 152–164.
- Levo, A. and Partanen, J. (1997a) Mutation-haplotype analysis of steroid 21-hydroxylase (CYP21) deficiency in Finland. Implications for the population history of defective alleles. *Hum. Genet.* **99**, 488–497.
- Levo, A. and Partanen, J. (1997b) Novel nonsense mutation (W302X) in the steroid 21-hydroxylase gene of a Finnish patient with the salt-wasting form of congenital adrenal hyperplasia. *Human Mutation* **9**, 363–365.
- Lewis, D.F. and Lee-Robichaud, P. (1998) Molecular modelling of steroidogenic cytochromes P450 from families CYP11, CYP17, CYP19 and CYP21 based on the CYP102 crystal structure. *J. Steroid Biochem. Molec. Biol.* **66**, 217–233.
- Li, Y. and Lau, L.F. (1997) Adrenocorticotrophic hormone regulates the activities of the orphan nuclear receptor Nur77 through modulation of phosphorylation. *Endocrinology* **138**, 4138–4146.
- Lobato, M.N., Aledo, R. and Meseguer, A. (1998) High variability of CYP21 gene rearrangements in Spanish patients with classic form of congenital adrenal hyperplasia. *Hum. Hered.* **48**, 216–225.
- Lobato, M.N., Ordóñez-Sánchez, M.L., Tusie-Luna, M.T. and Meseguer, A. (1999) Mutation analysis in patients with congenital adrenal hyperplasia in the Spanish population: identification of putative novel steroid 21-hydroxylase deficiency alleles associated with the classic form of the disease. *Hum. Hered.* **49**, 169–175.
- Matteson, K.J., Phillips, J.A., Miller, W.L., Chung, B.C., Orlando, P.J., Frisch, H., Ferrandez, A. and Burr, I.M. (1987) P450XXI (steroid 21-hydroxylase) gene deletions are not found in family studies of congenital adrenal hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5858–5862.
- Mercado, A.B., Wilson, R.C., Cheng, K.C., Wei, J.Q. and New, M.I. (1995) Prenatal treatment and diagnosis of congenital adrenal hyperplasia owing to steroid 21-hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **80**, 2014–2020.
- Milstone, D.S., Shaw, S.K., Parker, K.L., Szyf, M. and Seidman, J.G. (1992) An element regulating adrenal-specific steroid 21-hydroxylase expression is located within the slp gene. *J. Biol. Chem.* **267**, 21924–21927.
- Monier, S., Van Luc, P., Kreibich, G., Sabatini, D.D. and Adesnik, M. (1988) Signals for the incorporation and orientation of cytochrome P450 in the endoplasmic reticulum membrane. *J. Cell Biol.* **107**, 457–470.

- Morel, Y., Andre, J., Uring-Lambert, B., Hauptman, G., Betuel, H., Tossi, M., Forest, M., David, M., Bertrand, J. and Miller, W.L. (1989) Rearrangements and point mutations of P450c21 genes are distinguished by five restriction endonuclease haplotypes identified by a new probing strategy in 57 families with congenital adrenal hyperplasia. *J. Clin. Invest.* **83**, 527–536.
- Morley, S.D., Viard, I., Chung, B.C., Ikeda, Y., Parker, K.L. and Mullins, J.J. (1996) Variegated expression of a mouse steroid 21-hydroxylase/beta-galactosidase transgene suggests centripetal migration of adrenocortical cells. *Mol. Endocrinol.* **10**, 585–598.
- Mornet, E., Couillin, P., Kuttan, F., Raux, M.C., White, P.C., Cohen, D., Boue, A. and Dausset, J. (1986) Associations between restriction fragment length polymorphisms detected with a probe for human 21-hydroxylase (21-OH) and two clinical forms of 21-OH deficiency. *Hum. Genet.* **74**, 402–408.
- Mornet, E., Crete, P., Kuttann, F., Raux-Demay, M.C., Boue, J., White, P.C. and Boue, A. (1991) Distribution of deletions and seven point mutations on CYP21B genes in three clinical forms of steroid 21-hydroxylase deficiency. *Am. J. Hum. Genet.* **48**, 79–88.
- Narasimhulu, S. (1991) Inhibition of substrate binding to the adrenal cytochrome P450C-21 by acrylamide and its implications for solvent accessibility of the binding site in the microsomes. *Biochemistry* **30**, 9319–9327.
- Nelson, D.R. and Strobel, H.W. (1989) Secondary structure prediction of 52 membrane-bound cytochromes P450 shows a strong structural similarity to P450cam. *Biochemistry* **28**, 656–660.
- Nikoshkov, A., Lajic, S., Holst, M., Wedell, A. and Luthman, H. (1997) Synergistic effect of partially inactivating mutations in steroid 21-hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **82**, 194–199.
- Nikoshkov, A., Lajic, S., Vlamis-Gardikas, A., Tranebjaerg, L., Holst, M., Wedell, A. and Luthman, H. (1998) Naturally occurring mutants of human steroid 21-hydroxylase (P450c21) pinpoint residues important for enzyme activity and stability. *J. Biol. Chem.* **273**, 6163–6165.
- Nimkarn, S., Cerame, B.I., Wei, J.Q., Domic, M., Zunec, R., Brkljacic, L., Skrabic, V., New, M.I. and Wilson, R.C. (1999) Congenital adrenal hyperplasia (21-hydroxylase deficiency) without demonstrable genetic mutations. *J. Clin. Endocrinol. Metab.* **84**, 378–381.
- O'Neill, G.J., Dupont, B., Pollack, M.S., Levine, L.S. and New, M.I. (1982) Complement C4 allotypes in congenital adrenal hyperplasia due to 21-hydroxylase deficiency: further evidence for different allelic variants at the 21-hydroxylase locus. *Clin. Immunol. Immunopathol.* **23**, 312–322.
- Oelkers, W.K. (1996) Effects of estrogens and progestogens on the renin-aldosterone system and blood pressure. [Review]. *Steroids* **61**, 166–171.
- Ohlsson, G. and Schwartz, M. (1997) Mutations in the gene encoding 21-hydroxylase detected by solid-phase minisequencing. *Hum. Genet.* **99**, 98–102.
- Ordenez-Sanchez, M.L., Ramirez-Jimenez, S., Lopez-Gutierrez, A.U., Riba, L., Gamboa-Cardiel, S., Cerrillo-Hinojosa, M., Altamirano-Bustamante, N., Calzada-Leon, R., Robles-Valdes, C., Mendoza-Morfin, F. and Tusie-Luna, M.T. (1998) Molecular genetic analysis of patients carrying steroid 21-hydroxylase deficiency in the Mexican population: identification of possible new mutations and high prevalence of apparent germ-line mutations. *Hum. Genet.* **102**, 170–177.
- Owerbach, D., Ballard, A.L. and Draznin, M.B. (1992a) Salt-wasting congenital adrenal hyperplasia: detection and characterization of mutations in the steroid 21-hydroxylase gene, CYP21, using the polymerase chain reaction. *J. Clin. Endocrinol. Metab.* **74**, 553–558.
- Owerbach, D., Crawford, Y.M. and Draznin, M.B. (1990) Direct analysis of CYP21B genes in 21-hydroxylase deficiency using polymerase chain reaction amplification. *Mol. Endocrinol.* **4**, 125–131.

- Owerbach, D., Draznin, M.B., Carpenter, R.J. and Greenberg, F. (1992b) Prenatal diagnosis of 21-hydroxylase deficiency congenital adrenal hyperplasia using the polymerase chain reaction. *Hum. Genet.* **89**, 109–110.
- Owerbach, D., Sherman, L., Ballard, A.L. and Azziz, R. (1992c) Pro-453 to Ser mutation in CYP21 is associated with nonclassic steroid 21-hydroxylase deficiency. *Mol. Endocrinol.* **6**, 1211–1215.
- Parker, K.L. and Schimmer, B.P. (1997) Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr. Rev.* **18**, 361–377.
- Picado-Leonard, J. and Miller, W.L. (1988) Homologous sequences in Steroidogenic enzymes, steroid receptors and a steroid binding protein suggest a consensus steroid-binding sequence. *Mol. Endocrinol.* **2**, 1145–1150.
- Pollack, M.S., Levine, L.S., O'Neill, G.J., Pang, S., Lorenzen, F., Kohn, B., Rondonini, G.F., Chiumello, G., New, M.I. and Dupont, B. (1981) HLA linkage and B14, DR1, BfS haplotype association with the genes for late onset and cryptic 21-hydroxylase deficiency. *Am. J. Hum. Genet.* **33**, 540–550.
- Poulos, T.L. (1991) Modeling of mammalian P450s on basis of P450cam X-ray structure. *Methods Enzymol.* **206**, 11–30.
- Quigley, C.A., De Bellis, A., Marschke, K.B., el-Awady, M.K., Wilson, E.M. and French, F.S. (1995) Androgen receptor defects: historical, clinical, and molecular perspectives [published erratum appears in *Endocr. Rev.* 1995 Aug; 16 (4):546]. [Review]. *Endocr. Rev.* **16**, 271–321.
- Raux-Demay, M., Mornet, E., Boue, J., Couillin, P., Oury, J.F., Ravise, N., Deluchat, C. and Boue, A. (1989) Early prenatal diagnosis of 21-hydroxylase deficiency using amniotic fluid 17-hydroxyprogesterone determination and DNA probes. *Prenat. Diagn.* **9**, 457–466.
- Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J. (1993) Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* **261**, 731–736.
- Rein, H. and Jung, C. (1993) Metabolic reactions: mechanisms of substrate oxygenation. In: Schenkman, J.B. and Greim, H. (eds), *Cytochrome P450*, Springer-Verlag, Berlin, pp. 105–122.
- Reindollar, R.H., Lewis, J.B., White, P.C., Fernhoff, P.M., McDonough, P.G. and Whitney, J.B. (1988) Prenatal diagnosis of 21-hydroxylase deficiency by the complementary deoxyribonucleic acid probe for cytochrome P-450C-21 OH [published erratum appears in *Am. J. Obstet. Gynecol.* 1988 June; 158 (6 Pt 1):1445]. *Am. J. Obstet. Gynecol.* **158**, 545–547.
- Rice, D.A., Kronenberg, M.S., Mouw, A.R., Aitken, L.D., Franklin, A., Schimmer, B.P. and Parker, K.L. (1990) Multiple regulatory elements determine adrenocortical expression of steroid 21-hydroxylase. *J. Biol. Chem.* **265**, 8052–8058.
- Rodrigues, N.R., Dunham, I., Yu, C.Y., Carroll, M.C., Porter, R.R. and Campbell, R.D. (1987) Molecular characterization of the HLA-lined steroid 21-hydroxylase B gene from an individual with congenital adrenal hyperplasia. *EMBO J.* **6**, 1653–1661.
- Rumsby, G., Avey, C.J., Conway, G.S. and Honour, J.W. (1998) Genotype-phenotype analysis in late onset 21-hydroxylase deficiency in comparison to the classical forms. *Clin. Endocrinol. (Oxf)* **48**, 707–711.
- Rumsby, G., Carroll, M.C., Porter, R.R., Grant, D.B. and Hjelm, M. (1986) Deletion of the steroid 21-hydroxylase and complement C4 genes in congenital adrenal hyperplasia. *J. Med. Genet.* **23**, 204–209.
- Rumsby, G., Fielder, A.H., Hague, W.M. and Honour, J.W. (1988) Heterogeneity in the gene locus for steroid 21-hydroxylase deficiency. *J. Med. Genet.* **25**, 596–599.

- Rumsby, G., Honour, J.W. and Rodeck, C. (1993) Prenatal diagnosis of congenital adrenal hyperplasia by direct detection of mutations in the steroid 21-hydroxylase gene. *Clin. Endocrinol. (Oxf)* **38**, 421–425.
- Rumsby, G., Massoud, A.F., Avey, C. and Brook, C.G. (1996) Non-expression of a common mutation in the 21-hydroxylase gene: implications for prenatal diagnosis and carrier testing. *J. Med. Genet.* **33**, 798–799.
- Schneider, P.M., Carroll, M.C., Alper, C.A., Rittner, C., Whitehead, A.S., Yunis, E.J. and Colten, H.R. (1986) Polymorphism of the human complement C4 and steroid 21-hydroxylase genes. *J. Clin. Invest.* **78**, 650–657.
- Schulze, E., Scharer, G., Rogatzki, A., Priebe, L., Lewwicka, S., Bettendorf, M., Hoepffner, W., Heinrich, U.E. and Schwabe, U. (1995) Divergence between genotype and phenotype in relatives of patients with the intron 2 mutation of steroid 21-hydroxylase. *Endocr. Res.* **21**, 359–364.
- Seckl, J.R. and Miller, W.L. (1997) How safe is longterm prenatal glucocorticoid treatment? *JAMA* **277**, 1077–1079.
- Sevrioukova, I.F. and Peterson, J.A. (1995) NADPH-P-450 reductase: structural and functional comparisons of the eukaryotic and prokaryotic isoforms. [Review]. *Biochimie* **77**, 562–572.
- Shannon, M.F., Pell, L.M., Lenardo, M.J., Kuczek, E.S., Occhiodoro, F.S., nn, S.M. and Das, M.A. (1990) A novel tumor necrosis factor-responsive transcription factor which recognizes a regulatory element in hemopoietic growth factor genes. *Mol. Cell Biol.* **10**, 2950–2959.
- Shen, L., Wu, L.C., Sanlioglu, S., Chen, R., Mendoza, A.R., Dangel, A.W., Carroll, M.C., Zipf, W.B. and Yu, C.Y. (1994) Structure and genetics of the partially duplicated gene RP located immediately upstream of the complement C4A and the C4B genes in the HLA class III region. Molecular cloning, exon-intron structure, composite retroposon, and breakpoint of gene duplication. *J. Biol. Chem.* **269**, 8466–8476.
- Sherman, S.L., Aston, C.E., Morton, N.E., Speiser, P.W. and New, M.I. (1988) A segregation and linkage study of classical and nonclassical 21-hydroxylase deficiency. *Am. J. Hum. Genet.* **42**, 830–838.
- Shimizu, T., Tateishi, T., Hatano, M. and Fujii-Kuriyama, Y. (1991) Probing the role of lysines and arginines in the catalytic function of cytochrome P450d by site-directed mutagenesis. Interaction with NADPH-cytochrome P450 reductase. *J. Biol. Chem.* **266**, 3372–3375.
- Shiroishi, T., Sagai, T., Natsuume-Sakai, S. and Moriwaki, K. (1987) Lethal deletion of the complement component C4 and steroid 21-hydroxylase genes in the mouse H-2 class III region, caused by meiotic recombination. *Proc. Natl Acad. Sci. U.S.A.* **84**, 2819–2823.
- Siegel, S.F., Hoffman, E.P. and Trucco, M. (1994) Molecular diagnosis of 21-hydroxylase deficiency: Detection of four mutations on a single gel. *Biochem. Med. Metab. Biol.* **51**, 66–73.
- Sinnott, P., Collier, S., Costigan, C., Dyer, P.A., Harris, R. and Strachan, T. (1990) Genesis by meiotic unequal crossover of a *de novo* deletion that contributes to steroid 21-hydroxylase deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2107–2111.
- Speiser, P.W., Agdere, L., Ueshiba, H., White, P.C. and New, M.I. (1991) Aldosterone synthesis in salt-wasting congenital adrenal hyperplasia with complete absence of adrenal 21-hydroxylase. *N. Engl. J. Med.* **324**, 145–149.
- Speiser, P.W., Dupont, B., Rubinstein, P., Piazza, A., Kastelan, A. and New, M.I. (1985) High frequency of nonclassical steroid 21-hydroxylase deficiency. *Am. J. Hum. Genet.* **37**, 650–667.
- Speiser, P.W., Dupont, J., Zhu, D., Serrat, J., Buegeleisen, M., Tusie-Luna, M.T., Lesser, M., New, M.I. and White, P.C. (1992a) Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J. Clin. Invest.* **90**, 584–595.
- Speiser, P.W., Laforgia, N., Kato, K., Pareira, J., Khan, R., Yang, S.Y., Whorwood, C., White, P.C., Elias, S., Schriock, E. *et al.* (1990) First trimester prenatal treatment and molecular genetic

- diagnosis of congenital adrenal hyperplasia (21-hydroxylase deficiency) . *J. Clin. Endocrinol. Metab.* **70**, 838–848.
- Speiser, P.W. and New, M.I. (1987) Genotype and hormonal phenotype in nonclassical 21-hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **64**, 86–91.
- Speiser, P.W., New, M.I., Tannin, G.M., Pickering, D., Yang, S.Y. and White, P.C. (1992b) Genotype of Yupik Eskimos with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Hum. Genet.* **88**, 647–648.
- Speiser, P.W., New, M.I. and White, P.C. (1988) Molecular genetic analysis of nonclassic steroid 21-hydroxylase deficiency associated with HLA-B14, DR1. *N. Engl. J. Med.* **319**, 19–23.
- Speiser, P.W. and White, P.C. (1998) Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency. *Clin. Endocrinol. (Oxf.)* **49**, 411–417.
- Stoner, E., Dimartino-Nardi, J., Kuhnle, U., Levine, L.S., Oberfield, S.E. and New, M.I. (1986) Is salt-wasting in congenital adrenal hyperplasia due to the same gene as the fasciculata defect? *Clin. Endocrinol. (Oxf.)* **24**, 9–20.
- Strumberg, D., Hauffa, B.P., Horsthemke, B. and Grosse-Wilde, H. (1992) Molecular detection of genetic defects in congenital adrenal hyperplasia due to 21-hydroxylase deficiency: a study of 27 families. *Eur. J. Pediatr.* **151**, 821–826.
- Suto, Y., Tokunaga, K., Watanabe, Y. and Hirai, M. (1996) Visual demonstration of the organization of the human complement C4 and 21-hydroxylase genes by high-resolution fluorescence *in situ* hybridization. *Genomics* **33**, 321–324.
- Swain, A., Narvaez, V., Burgoyne, P., Camerino, G. and Lovell-Badge, R. (1998) Daxl antagonizes Sry action in mammalian sex determination. *Nature* **391**, 761–767.
- Tajima, T., Fujieda, K., Nakayama, K. and Fujii-Kuriyama, Y. (1993) Molecular analysis of patient and carrier genes with congenital steroid 21-hydroxylase deficiency by using polymerase chain reaction and single strand conformation polymorphism. *J. Clin. Invest.* **92**, 2182–2190.
- Tee, M.K., Babalola, G.O., Aza-Blanc, P., Speek, M., Gitelman, S.E. and Miller, W.L. (1995a) A promoter within intron 35 of the human C4A gene initiates abundant adrenal-specific transcription of a 1kb RNA: location of a cryptic CYP21 promoter element? *Hum. Mol. Genet.* **4**, 2109–2116.
- Tee, M.K., Thomson, A.A., Bristow, J. and Miller, W.L. (1995b) Sequences promoting the transcription of the human XA gene overlapping P450c21A correctly predict the presence of a novel, adrenal-specific, truncated form of tenascin-X. *Genomics* **28**, 171–178.
- Tusie-Luna, M.T., Speiser, P.W., Dumez, M., New, M.I. and White, P.C. (1991) A mutation (Pro-30 to Leu) in CYP21 represents a potential nonclassic steroid 21-hydroxylase deficiency allele. *Mol. Endocrinol.* **5**, 685–692.
- Tusie-Luna, M.T., Traktman, P. and White, P.C. (1990) Determination of functional effects of mutations in the steroid 21-hydroxylase gene (CYP21) using recombinant vaccinia virus. *J. Biol. Chem.* **265**, 20916–20922.
- Tusie-Luna, M.T. and White, P.C. (1995) Gene conversions and unequal crossovers between CYP21 (steroid 21-hydroxylase gene) and CYP21P involve different mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10796–10800.
- Urabe, K., Kimura, A., Harada, F., Iwanaga, T. and Sasazuki, T. (1990) Gene conversion in steroid 21-hydroxylase genes. *Am. J. Hum. Genet.* **46**, 1178–1186.
- Wada, A. and Waterman, M.R. (1992) Identification by site-directed mutagenesis of two lysine residues in cholesterol side chain cleavage cytochrome P450 that are essential for adrenodoxin binding. *J. Biol. Chem.* **267**, 22877–22882.
- Waterman, M.R. and Bischof, L.J. (1997) Cytochromes P450 12: diversity of ACTH (cAMP)-dependent transcription of bovine steroid hydroxylase genes. [Review]. *FASEB J.* **11**, 419–427.

- Wedell, A. (1998) Molecular genetics of congenital adrenal hyperplasia (21-hydroxylase deficiency): implications for diagnosis, prognosis and treatment. [Review]. *Acta Paediat.* **87**, 159–164.
- Wedell, A. and Luthman, H. (1993a) Steroid 21-hydroxylase (P450c21): a new allele and spread of mutations through the pseudogene. *Hum. Genet.* **91**, 236–240.
- Wedell, A. and Luthman, H. (1993b) Steroid 21-hydroxylase deficiency: two additional mutations in salt-wasting disease and rapid screening of disease-causing mutations. *Hum. Mol. Genet.* **2**, 499–504.
- Wedell, A., Ritzen, E.M., Haglund-Stengler, B. and Luthman, H. (1992) Steroid 21-hydroxylase deficiency: three additional mutated alleles and establishment of phenotype-genotype relationships of common mutations. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7232–7236.
- Wedell, A., Thilen, A., Ritzen, E.M., Stengler, B. and Luthman, H. (1994) Mutational spectrum of the steroid 21-hydroxylase gene in Sweden: implications for genetic diagnosis and association with disease manifestations. *J. Clin. Endocrinol. Metab.* **78**, 1145–1152.
- Werkmeister, J.W., New, M.I., Dupont, B. and White, P.C. (1986) Frequent deletion and duplication of the steroid 21-hydroxylase genes. *Am. J. Hum. Genet.* **39**, 461–469.
- White, P.C. (1987) Genetics of steroid 21-hydroxylase deficiency. *Rec. Prog. Horm. Res.* **43**, 305–336.
- White, P.C. (1994) Disorders of aldosterone biosynthesis and action. *N. Engl. J. Med.* **331**, 250–258.
- White, P.C., Chaplin, D.D., Weis, J.H., Dupont, B., New, M.I. and Seidman, J.G. (1984a) Two steroid 21-hydroxylase genes are located in the murine S region. *Nature* **312**, 465–467.
- White, P.C., Grossberger, D., Onufer, B.J., Chaplin, D.D., New, M.I., Dupont, B. and Strominger, J.L. (1985) Two genes encoding steroid 21-hydroxylase are located near the genes encoding the fourth component of complement in man. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1089–1093.
- White, P.C., New, M.I. and Dupont, B. (1984b) HLA-linked congenital adrenal hyperplasia results from a defective gene encoding a cytochrome P-450 specific for steroid 21-hydroxylation. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7505–7509.
- White, P.C., New, M.I. and Dupont, B. (1986) Structure of human steroid 21-hydroxylase genes. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5111–5115.
- White, P.C., Vitek, A., Dupont, B. and New, M.I. (1988) Characterization of frequent deletions causing steroid 21-hydroxylase deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4436–4440.
- Wilson, J.D., Griffin, J.E. and Russell, D.W. (1993) Steroid 5 alpha-reductase 2 deficiency. [Review]. *Endocr. Rev.* **14**, 577–593.
- Wilson, R.C., Mercado, A.B., Cheng, K.C. and New, M.I. (1995a) Steroid 21-hydroxylase deficiency: genotype may not predict phenotype. *J. Clin. Endocrinol. Metab.* **80**, 2322–2329.
- Wilson, R.C., Wei, J.Q., Cheng, K.C., Mercado, A.B. and New, M.I. (1995b) Rapid deoxyribonucleic acid analysis by allele-specific polymerase chain reaction for detection of mutations in the steroid 21-hydroxylase gene. [Review]. *J. Clin. Endocrinol. Metab.* **80**, 1635–1640.
- Wilson, T.E., Mouw, A.R., Weaver, C.A., Milbrandt, J. and Parker, K.L. (1993) The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Mol. Cell Biol.* **13**, 861–868.
- Witchel, S.F., Bhamidipati, O.K., Hoffman, E.P. and Cohen, J.B. (1996) Phenotypic heterogeneity associated with the splicing mutation in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **81**, 4081–4088.

- Wu, D.A. and Chung, B.C. (1991) Mutations of P450c21 (steroid 21-hydroxylase) at Cys428, Val281, and Ser268 result in complete, partial, or no loss of enzymatic activity, respectively. *J. Clin. Invest.* **88**, 519–523.
- Wu, D.A., Hu, M.C. and Chung, B.C. (1991) Expression and functional study of wild-type and mutant human cytochrome P450c21 in *Saccharomyces cerevisiae*. *DNA Cell Biol.* **10**, 201–209.
- Yoshioka, H., Morohashi, K., Sogawa, K., Yamane, M., Kominami, S., Takemori, S., Okada, Y., Omura, T. and Fujii-Kuriyama, Y. (1986) Structural analysis of cloned cDNA for mRNA of microsomal cytochrome P-450(C21) which catalyzes steroid 21-hydroxylation in bovine adrenal cortex. *J. Biol. Chem.* **261**, 4106–4109.
- Zhang, W.J., Degli-Esposti, M.A., Cobain, T.J., Cameron, P.U., Christiansen, F.T. and Dawkins, R.L. (1990) Differences in gene copy number carried by different MHC ancestral haplotypes. Quantitation after physical separation of haplotypes by pulsed field gel electrophoresis. *J. Exp. Med.* **171**, 2101–2114.

## 7.

# STEROID 11 $\beta$ -HYDROXYLASE ISOZYMES

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Both glucocorticoids and mineralocorticoids require 11 $\beta$ -hydroxylation for full activity. Disorders of 11 $\beta$ -hydroxylation in the adrenal cortex affect the biosynthesis of these hormones and thus have significant physiological effects. The genetics of enzymes required for steroid 11 $\beta$ -hydroxylase are reviewed in this chapter.

KEY WORDS: 11 $\beta$ -hydroxylase cytochrome P450, aldosterone synthase, congenital adrenol hyperplasia, salt-wasting, hypertension.

## BIOCHEMISTRY OF ADRENAL STEROID BIOSYNTHESIS

### Zonal location of steroid biosynthesis

Cortisol is synthesized from cholesterol in the zona fasciculata of the adrenal cortex. This process requires five enzymatic conversions (see [Figure 6.1](#) in the previous chapter): cleavage of the cholesterol side-chain to yield pregnenolone, 17 $\alpha$ -hydroxylation and 3 $\beta$ -dehydrogenation to 17-hydroxyprogesterone, and successive hydroxylations at the 21 and 11 $\beta$  positions. A “17-deoxy” pathway is also active in the zona fasciculata, in which 17 $\alpha$ -hydroxylation does not occur and the final product is normally corticosterone.

The same 17-deoxy pathway is active in the adrenal zona glomerulosa, which contains no 17 $\alpha$ -hydroxylase activity. However, corticosterone is not the final product in the zona glomerulosa; instead, corticosterone is successively hydroxylated and oxidized at the 18 position to yield aldosterone.

The need for functional zonation of the adrenal gland is apparent when one considers that the amount of aldosterone needed to control salt balance is 100–1000 fold less than the amount of cortisol needed to control carbohydrate metabolism. The necessary precursors for aldosterone biosynthesis (in particular, progesterone and deoxycorticosterone) are also synthesized in the much larger zona fasciculata at levels that would lead to mineralocorticoid excess if they were converted to aldosterone. The zonation of the adrenal is therefore one way to control the relative production rates of mineralocorticoid and glucocorticoid.



### Steroid 11 $\beta$ -hydroxylase isozymes

Humans have two 11 $\beta$ -hydroxylase isozymes that are respectively responsible for cortisol and aldosterone biosynthesis, CYP11B1 (also termed 11 $\beta$ -hydroxylase, P450c11, P450XIB1) and CYP11B2 (aldosterone synthase, also termed P450aldo, P450c18, P450cmo, P450XIB2). These isozymes are mitochondrial cytochromes P450 located in the inner membrane on the matrix side. Each is synthesized with 503 amino acid residues, but a signal peptide is cleaved in mitochondria to yield the mature protein of 479 residues (Yanagibashi *et al.*, 1986). The sequences of the proteins are 93% identical (Mornet *et al.*, 1989). Although CYP11B1 and CYP11B2 should be identical in size based on their amino acid sequences, they have apparent molecular weights of 51 and 49 kD respectively as determined by SDS-polyacrylamide gel electrophoresis (Curnow *et al.*, 1991; Ogishima *et al.*, 1991). The reason for this difference is not known.

The CYP11B1 isozyme 11 $\beta$ -hydroxylates 11-deoxycortisol to cortisol, as determined by expressing the corresponding cDNA in cultured cells (Kawamoto *et al.*, 1990; Curnow *et al.*, 1991; Kawamoto *et al.*, 1992) and after actual purification from aldosterone-secreting tumors (Ogishima *et al.*, 1991). It can also convert 11-deoxycorticosterone to either corticosterone (the predominant product) or 18-hydroxy-11-deoxycorticosterone, but it 18-hydroxylates corticosterone poorly. It cannot convert corticosterone into aldosterone. In contrast, CYP11B2 has somewhat weaker 11 $\beta$ -hydroxylase activity than CYP11B1 but it can 18-hydroxylate and then 18-oxidize corticosterone to aldosterone. When deoxycorticosterone is converted to aldosterone, the same steroid molecule probably remains bound to the enzyme for all three conversions without release of the intermediate products.

CYP11B2 can also convert 11-deoxycortisol to 18-oxocortisol. This reaction normally does not occur at significant levels *in vivo* because CYP11B2 is not expressed in the zona fasciculata, but it becomes important in patients with glucocorticoid-suppressible hyperaldosteronism (see below).

Although the human CYP11B1 and CYP11B2 enzymes differ in 33 amino acid residues, studies of chimeric enzymes from patients with glucocorticoid-suppressible aldosteronism (see below) suggested that the residues specific to CYP11B2 that are critical for 18-hydroxylase and 18-oxidase activities are located in the C-terminal half of the enzyme (Pascos *et al.*, 1992a). It now appears that these residues include Gly-288 (Ser in CYP11B1), Ala-320 (Val in CYP11B1) and Asp-335 (Asn in CYP11B1) (Bottner *et al.*, 1998; Mulatero *et al.*, 1998).

### Catalytic mechanism

Like other P450 enzymes, CYP11B1 and CYP11B2 utilize molecular oxygen and reducing equivalents (i.e., electrons) provided by NADPH to catalyze specific hydroxylations. As

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with other mitochondrial P450s, the reducing equivalents are not accepted directly from NADPH but instead from a series of two accessory electron transport proteins; adrenodoxin reductase accepts electrons from NADPH and donates them to adrenodoxin which in turn transfers them to the P450. These intermediate proteins are necessary because NADPH donates electrons in pairs whereas P450s can only accept single electrons. Adrenodoxin (also termed ferredoxin) contains non-heme iron complexed with sulfur. Adrenodoxin (ferredoxin) reductase is a flavoprotein that contains a single molecule of FAD. Only one gene encoding each accessory protein has been documented in mammals (Solish *et al.*, 1988; Picado-Leonard *et al.*, 1988).

The interaction between the P450 and adrenodoxin requires basic amino acid residues on the P450 and acidic residues on adrenodoxin. Based on mutagenesis studies in the related enzyme, CYP11A (Wada and Waterman, 1992) and X-ray crystallographic studies of bacterial P450 enzymes (Poulos, 1991; Ravichandran *et al.*, 1993), Arg-366 and Lys-370 in the CYP11B isozymes are likely to be important for this interaction.

Catalytically important residues include Cys-450, which is the fifth ligand of the heme iron atom (Nelson and Strobel, 1989) and Thr-318, which donates protons to bound molecular oxygen (Ravichandran *et al.*, 1993).

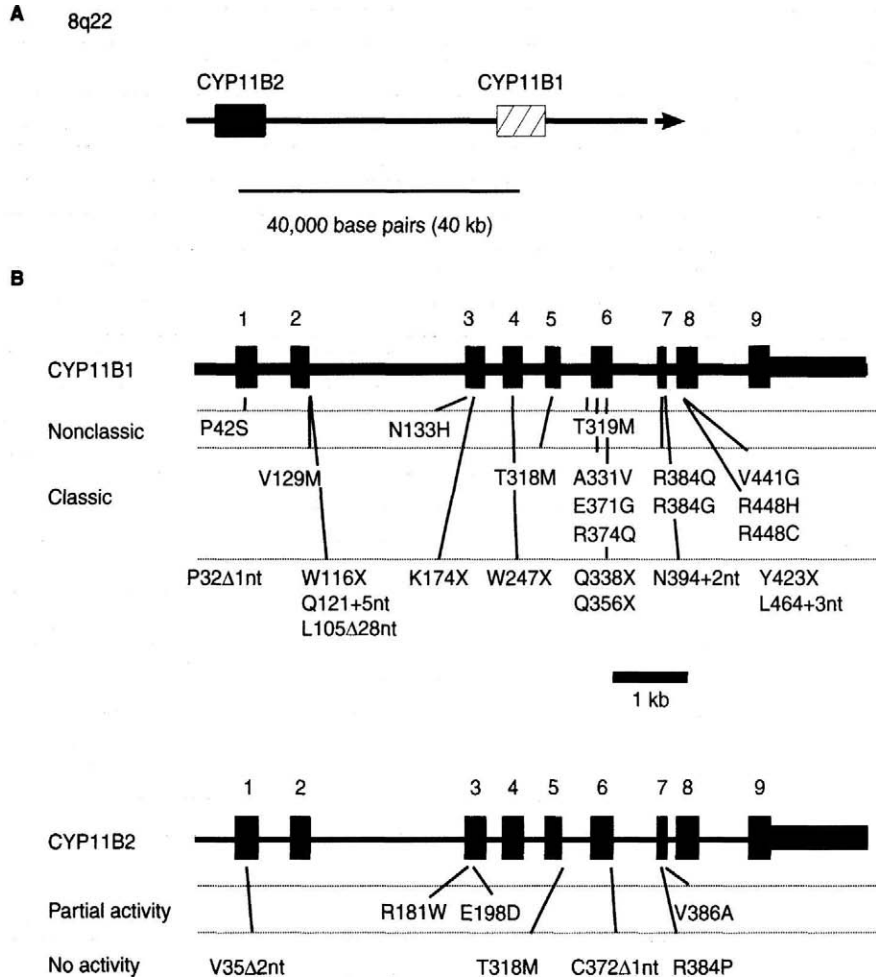
## GENETICS OF STEROID 11 $\beta$ -HYDROXYLASE ISOZYMES

### Characteristics of the human CYP11B genes

In humans, CYP11B1 and CYP11B2 are encoded by two genes (Mornet *et al.*, 1989) on chromosome 8q24.3 (Taymans *et al.*, 1998; Wagner *et al.*, 1991)(Figure 7.1). Each contains nine exons spread over approximately 7,000 base pairs (7kb) of DNA. The nucleotide sequences of these genes are 95% identical in coding sequences and about 90% identical in introns. The predicted amino acid sequences are 36% identical to those of cholesterol desmolase (CYP11A), another mitochondrial P450. Moreover, the locations of introns within the coding sequences of CYP11A, CYP11B1 and CYP11B2 are identical. For these reasons, these genes are grouped into a single family within the cytochrome P450 gene superfamily (Nelson *et al.*, 1993).

The human CYP11B1 and CYP11B2 genes are located approximately 40Kb apart (Pascoe *et al.*, 1992a; Lifton *et al.*, 1992b). CYP11B2 is on the left (if the genes are pictured as being transcribed left to right).

CYP11B1 is expressed at high levels in normal adrenal glands (Mornet *et al.*, 1989). CYP11B2 transcripts cannot be detected by hybridization to Northern blots of normal adrenal RNA (Mornet *et al.*, 1989), but such transcripts have been detected in normal adrenal RNA using reverse-transcription followed by the polymerase chain reaction (RT-PCR) (Curnow *et al.*, 1991). CYP11B2 transcripts are present at increased levels in aldosterone secreting tumors (Kawamoto *et al.*, 1990; Curnow *et al.*, 1991). Using *in situ* hybridization, CYP11B2 transcripts appear to be expressed only in the glomerulosa of all mammalian adrenals examined, whereas CYP11B1 is expressed predominantly in the zona fasciculata (Domalik *et al.*, 1991; Ogishima *et al.*, 1992; Pascoe *et al.*, 1995).



**Figure 7.1 Mutations involving the 11 $\beta$ -hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) genes.** (A) the arrangement of genes is diagramed. (B) mutations in CYP11B1 causing nonclassic or classic 11 $\beta$ -hydroxylase deficiency, and mutations in CYP11B2 causing aldosterone synthase deficiency. These are arranged in the diagram so that those causing increasing enzymatic compromise are arrayed from top to bottom. Dotted lines divide mutants into groups with similar activities. As an example of mutation terminology, P42S is Proline-42 to Serine.  $\Delta$ , deletion; +, insertion. Other single letter amino acid codes: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine. Although there are two types of aldosterone synthase deficiency, they do not correspond exactly to *in vitro* levels of enzymatic activity. R181W and V386A do not cause disease separately, but double homozygosity causes CMO II type aldosterone synthase deficiency. Other combinations with this phenotype include T318M+V386A and R181W+C372 $\Delta$ 1nt. Homozygosity for either V35 $\Delta$ 2nt and R384P, or double homozygosity for E198D+V386A, has been associated with CMO I type deficiency.

### Other mammalian species

Cattle, pigs and sheep have a single enzyme (Hall *et al.*, 1991; Boon *et al.*, 1998) that is expressed in both zones and synthesizes both cortisol and aldosterone; aldosterone synthesis in the zona fasciculata of cattle is suppressed by an unknown mechanism. This may involve differences in lipid composition of mitochondrial membranes in the two zones, or CYP11A (cholesterol desmolase), which is expressed at higher levels in the zona fasciculata, may compete for electrons with CYP11B and inhibit its 18-oxidase activity (Cao and Bernhardt, 1999).

Mice have distinct CYP11B1 and CYP11B2 isozymes active in the zona fasciculata and zona glomerulosa; only the latter has significant 18-hydroxylase and 18-oxidase activity (Domalik *et al.*, 1991).

Whereas mice have two CYP11B1 and CYP11B2 genes (Domalik *et al.*, 1991), the rat genome contains three independent CYP11B genes termed rCYP11B1, rCYP11B2, and rCYP11B3 (Nomura *et al.*, 1993). There also appears to be a fourth gene, rCYP11B4, which is considered a pseudogene. Sequence divergence in the amino acid sequence of the rat isozymes has allowed the production of specific antibodies for the rCYP11B1 and rCYP11B2 isozymes. rCYP11B1 represents the 11 $\beta$ -hydroxylase isozyme expressed in the fasciculata and rCYP11B2 the aldosterone synthase isozyme expressed in glomerulosa (Ogishima *et al.*, 1992). rCYP11B3 is expressed within the adrenal during fetal development, with expression lost after birth (Mellon *et al.*, 1995).

## REGULATION

The CYP11B1 and CYP11B2 genes are regulated in a manner consistent with their respective roles in cortisol and aldosterone biosynthesis.

### Regulation of cortisol biosynthesis

Cortisol synthesis is primarily controlled by ACTH (corticotropin) (Waterman and Simpson, 1989). ACTH acts through a specific G protein-coupled receptor on the surface of cells of the adrenal cortex (Mountjoy *et al.*, 1992), to increase levels of cAMP (adenosine 3', 5' monophosphate). Cyclic AMP has short-term (minutes to hours) effects on transport of cholesterol into mitochondria through increasing the synthesis of a short lived protein, steroidogenic acute regulatory (StAR) protein (Stocco and Clark, 1996a). It has longer term (hours to days) effects on transcription of genes encoding the enzymes required to synthesize cortisol including CYP11B1 (Waterman and Bischof, 1997), and preferentially increases CYP11B1 mRNA expression over that of CYP11B2 (Curnow *et al.*, 1991; Denner *et al.*, 1996).

### Regulation of aldosterone biosynthesis

The rate of aldosterone synthesis, which is normally 100–1000 fold less than that of cortisol synthesis, is regulated mainly by the renin-angiotensin system and by potassium levels with ACTH having only a short-term effect (Rainey and White, 1998). Because the necessary precursors for aldosterone biosynthesis (in particular, deoxycorticosterone) are also synthesized in the much larger zona fasciculata, it is apparent that there must be unique regulated steps in aldosterone biosynthesis in the zona glomerulosa or this process would simply be regulated by ACTH. These regulated steps seem to be those mediated by aldosterone synthase (CYP11B2) (Adler *et al.*, 1993).

#### *Angiotensin II*

*In vivo*, sodium restriction increases renin and angiotensin II levels. Renin is a proteolytic enzyme secreted by the juxtaglomerular apparatus of the nephron in response to decreased volume as sensed by stretch receptors in the afferent arteriole. Renin digests angiotensinogen to angiotensin I, a decapeptide which is converted by angiotensin converting enzyme to an octapeptide, angiotensin II. Angiotensin II occupies a G protein-coupled receptor, activating phospholipase C. The latter protein hydrolyses phosphatidylinositol biphosphate to produce inositol triphosphate and diacylglycerol, which raise intracellular calcium levels.

These increase glomerulosa CYP11B2 expression (Lauber *et al.*, 1990; Tremblay *et al.*, 1992; Holland *et al.*, 1993; Adler *et al.*, 1993). Under these conditions adrenal expression of CYP11B1 is not affected. The effects of angiotensin II can be inhibited by antagonists to the type 1 angiotensin II (AT1) receptor (Kakiki *et al.*, 1997). Angiotensin II receptors are predominantly expressed in the zona glomerulosa, suggesting a role for angiotensin II in the glomerulosa-specific expression of CYP11B2 (Breault *et al.*, 1996).

*In vitro*, adrenal AT1 receptors couple to several signaling pathways. The most characterized of these is the activation of phospholipase C, which increases intracellular calcium and diacylglycerol. These second messengers activate calmodulin and protein kinase C (PKC) respectively. PKC does not increase transcript levels of CYP11B2 or CYP11B1 and indeed is a potent inhibitor of the expression of CYP17 (Bird *et al.*, 1996). Thus, PKC activation may play an important role in the zonation of the adrenal by blocking expression of CYP17 in the glomerulosa.

Treatment of primary cultures of glomerulosa cells and H295R adrenal cells (Bird *et al.*, 1993) with angiotensin II rapidly increases intracellular calcium levels. The ability of both angiotensin II and  $K^+$  to increase intracellular calcium makes this cellular signaling pathway a likely common mechanism for the acute and chronic regulation of aldosterone biosynthesis. However, angiotensin II mediated increases in CYP11B2 mRNA levels are not sensitive to the inhibition of calmodulin or CaM kinase (Pezzi *et al.*, 1997) whereas expression of CYP11B2 reporter constructs is inhibited by the same agents. Thus, angiotensin II may

induce CYP11B2 or affect mRNA stability through signaling pathways in addition to intracellular calcium levels.

#### *Potassium*

Adrenal CYP11B2 expression is induced following potassium loading in rats. In mice with targeted deletions of genes in the renin/angiotensin system, K<sup>+</sup> can substitute for angiotensin II and increase CYP11B2 expression in the adrenal (Okubo *et al.*, 1997; Chen *et al.*, 1997). These *in vivo* data support *in vitro* studies demonstrating that K<sup>+</sup> stimulates expression of CYP11B2 both in primary cultures of rat glomerulosa and in human H295R adrenocortical cells (Bird *et al.*, 1995; Denner *et al.*, 1996). Potassium signaling in glomerulosa cells involves membrane depolarization leading to an influx of calcium through T and L-type channels. Consistent with this, elevating intracellular calcium with the calcium channel agonist BAYK8644 increases expression of CYP11B2 mRNA in H295R adrenal cells. Moreover, calcium channel blockers such as nifedipine block K<sup>+</sup> induction of CYP11B2 (Denner *et al.*, 1996; Pezzi *et al.*, 1997; Clyne *et al.*, 1997).

Intracellular calcium signaling often occurs through the action of the calcium binding protein, calmodulin. Calmodulin (CaM) is a widely expressed protein that, in its calcium-bound form, activates a variety of enzymes and kinases (Weinstein and Mehler, 1994). The tissue-specific expression of these kinases appears to be an important mechanism controlling the different tissue responses to this signaling pathway. Of the various CaM kinases described to date, the multifunctional family of CaM kinases (types I, II and IV) are most likely to be involved in angiotensin II and K<sup>+</sup> induction of aldosterone production. These kinases phosphorylate a wide variety of substrates and thus are distinct from the dedicated calcium activated kinases such as myosin light chain kinase or phosphorylase kinase. Antagonists of CaM and CaM-dependent protein kinase completely inhibit K<sup>+</sup> induction of CYP11B2 mRNA (Pezzi *et al.*, 1997). Thus, K<sup>+</sup> stimulation of chronic aldosterone secretion apparently relies on a relatively straightforward pathway from increased intracellular calcium signaling that activates calmodulin-dependent protein kinases, increasing expression of CYP11B2.

#### *Corticotropin (ACTH)*

The physiologic relevance of ACTH in aldosterone production and CYP11B2 transcription is unclear. Mice with targeted ablation of pituitary corticotropes can maintain adrenal expression of CYP11B2, but not CYP11B1, despite very low circulating ACTH (Allen *et al.*, 1995), a finding that confirms observations in humans with secondary adrenal insufficiency. Moreover, although ACTH causes an acute increase in the production of aldosterone both *in vivo* and in isolated cells, it acts chronically to decrease plasma aldosterone levels in humans and adrenal expression of CYP11B2 in animal models (Aguilera, 1993; Mitani *et al.*, 1996). Whereas ACTH chronically affects the expression of steroid hydroxylases mainly through

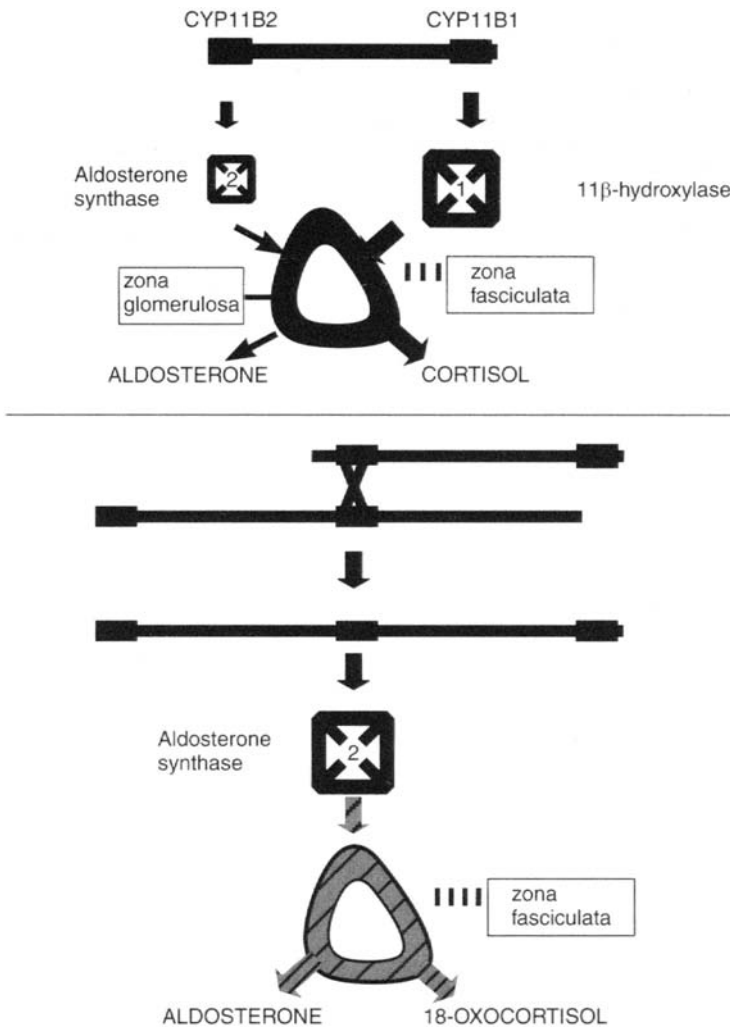
increases in intracellular cAMP levels, the mechanisms through which ACTH inhibits expression of CYP11B2 are uncertain. Cyclic AMP signaling has a negative effect on the expression of angiotensin II receptors in adrenocortical cells (Bird *et al.*, 1994) thereby desensitizing adrenal cells to angiotensin II (see below). ACTH might also decrease aldosterone synthesis by inducing CYP11B1 and CYP17, enzymes that effectively remove precursors from the pathway leading to mineralocorticoids and utilize them instead to synthesize cortisol (Bird *et al.*, 1996). The glomerulosa has additional mechanisms to control intracellular levels of cAMP generated by ACTH. First, in isolated glomerulosa cells but not in fasciculata cells, angiotensin II (through coupling to Gi) inhibits ACTH-stimulated cAMP production (Hausdorff *et al.*, 1987; Begeot *et al.*, 1988). Second, the adrenal glomerulosa (but not the fasciculata) expresses adenylyl cyclases (types 5 and 6) that are inhibited by intracellular calcium (Shen *et al.*, 1997), levels of which are increased by both angiotensin II and  $K^+$ .

### TRANSCRIPTIONAL REGULATION OF CYP11B1 AND CYP11B2

Although this chapter concentrates on human genetics, we first consider regulation of the murine and bovine genes because the data were obtained before human gene regulation had been extensively studied.

#### Bovine

Considerable work has been accomplished on the mechanisms that regulate transcription of the bovine CYP11B (bCYP11B) gene (Kawamoto *et al.*, 1990; Morohashi *et al.*, 1990; Honda *et al.*, 1990; Hashimoto *et al.*, 1992; Honda *et al.*, 1993; Takayama *et al.*, 1994). DNase I footprint analysis defines 6 protected regions in bCYP11B proximal DNA termed Adrenal (Ad) 1–6. Analyses of these putative *cis*-elements by transfection of reporter constructs in mouse Y-1 adrenocortical cells demonstrated a need for the Ad1 and Ad4 elements for basal and cAMP-stimulated expression of reporter activity. The Ad1 element closely resembled a consensus CRE (Figure 7.2) and bound similar proteins in nuclear extracts from steroidogenic and non-steroidogenic cells. These proteins are probably members of the CREB/ATF family. The Ad4 element binds a protein expressed predominantly in steroidogenic tissues termed Ad4-binding protein (Ad4BP) (Honda *et al.*, 1993) or steroidogenic factor-1 (SF-1) (Lala *et al.*, 1992). This protein is considered a key transcription factor in the regulation of a number of steroid hydroxylases as well as the steroidogenic acute regulatory (StAR) protein and 3 $\beta$ -hydroxysteroid dehydrogenase genes (Parker and Schimmer, 1997). While clearly important for transcription of bCYP11B, the high level of SF-1 expression in the gonads makes it unlikely that SF-1 alone determines the adrenal-specific expression of this gene. In addition, as noted above, the cow has only one CYP11B gene that is expressed in both the glomerulosa and fasciculata of the adrenal. Therefore, the information obtained from the study of the regulatory elements in the bCYP11B 5'-flanking DNA cannot completely



**Figure 7.2 (Top)** schematic of CYP11B1 and CYP11B2 genes, showing patterns of expression of each. **(Bottom)** unequal crossing over generating a chimeric CYP11B1/2 gene that has aldosterone synthase activity but is expressed in the zona fasciculata. This causes glucocorticoid suppressible hyperaldosteronism.

explain zone-specific expression of the multiple CYP11B genes seen in rodents and human. The ability of angiotensin II to stimulate production of aldosterone in fetal bovine adrenal cells suggests that it can influence the aldosterone synthase activity of CYP11B through unknown mechanisms (Rainey *et al.*, 1992).



### Murine

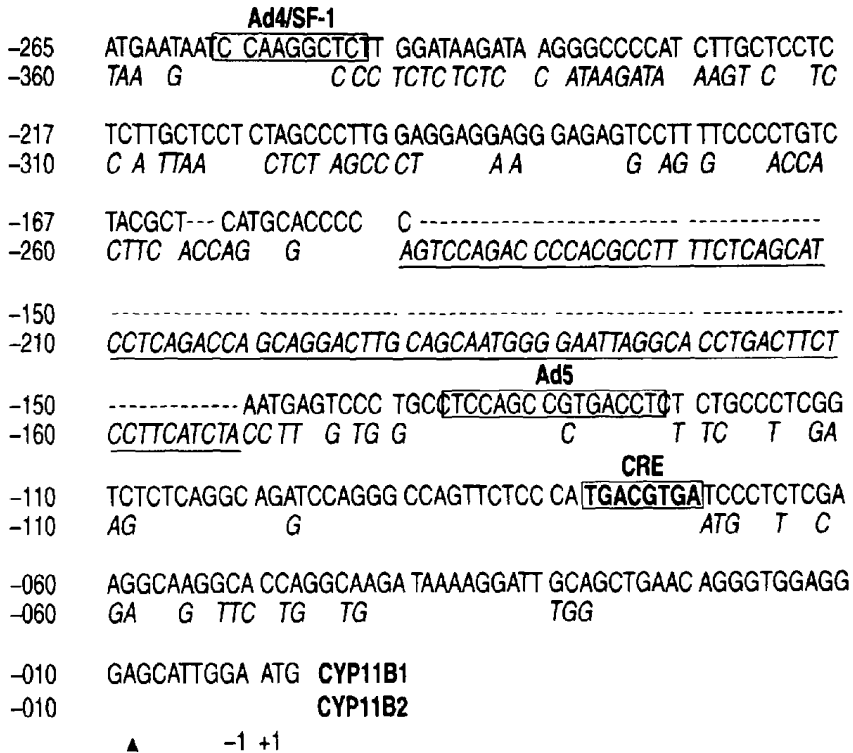
Deletion analysis, DNase I footprinting, gel-mobility shift assay and mutagenesis have defined several *cis* elements in the 5'-flanking DNA of the mouse CYP11B2 gene (Wong *et al.*, 1989). A CRE located at -76 is homologous to the Ad1 element found in bCYP11B and is essential for basal and cAMP-induced activity (Figure 7.2). Expression is dependent on activity of protein kinase A. Southwestern analysis demonstrated that a 43kDa protein from Y-1, HeLa and PC-12 nuclear extracts bound the CYP11B2 CRE. This molecular weight is similar to several members of the CREB/ATF family of transcription factors. Two elements are important for basal transcription, one at -397 and the other at -337. The -337 element is similar in sequence to the Ad4 element described in the bCYP11B gene and binds the same protein, SF-1 or Ad4BP (Lala *et al.*, 1992).

### Rat

The 5'-flanking sequences of rCYP11B1 and rCYP11B3 are highly similar (93%) whereas the flanking DNA of rCYP11B2 is less than 50% identical to the others. An AP-1 sequence in the rCYP11B1 flanking DNA may play an important role in transcription of this gene (Mukai *et al.*, 1995, 1998). AP-1 *cis*-elements are regulated by the expression of members of the *fos/jun* family of transcription factors. Using DNase I footprinting, gel mobility shift assays and mutagenesis these authors have shown that an AP-1 element that lies adjacent to the Ad4 element is important for rCYP11B1 reporter gene expression in Y-1 adrenal cells. Indeed mutation of the Ad4 site does not influence expression while mutation of the AP-1 site destroyed both basal and stimulated reporter activities. In addition, co-transfection of Y-1 cells with rCYP11B1 reporter construct and expression vector containing *c-jun* and *c-fos* enhanced reporter activity. Comparison between species suggests that a similar AP-1 element is present in human CYP11B1 but not in human CYP11B2; therefore it could play a role in zonal expression of these isozymes. In support of this premise, the authors went on to demonstrate that nuclei in the rat adrenal fasciculata express much higher levels of AP-1 binding proteins (*jun* and *fos*) than cells in the glomerulosa.

### Human

Initial attempts to define the *cis*-acting regulatory elements in the 5'-flanking region of CYP11B1 and CYP11B2 involved transfecting reporter constructs into mouse Y-1 adrenocortical cells, but CYP11B2 was expressed poorly in this system (Kawamoto *et al.*, 1992). Subsequent studies (Clyne *et al.*, 1997) have used H295R human adrenocortical cells, and these will be discussed in more detail. These cells are useful for studying the hCYP11B genes because they express both isozymes under the control of the presumptive physiologic signaling systems (cAMP, angiotensin II and K<sup>+</sup>). Deletion and mutation analyses of the 5'-flanking region of hCYP11B1 demonstrates the essential role of an Ad4/SF-1 element at -249 in SF-1 enhanced transcription (unpublished observations). This *cis*-regulatory element resembles the Ad4 element seen in other CYP11B genes studied to date (Figure 7.2). In addition, the CRE at position -78 is needed for cAMP stimulation of reporter activity. The CYP11B1 CRE has considerable sequence similarity to the Ad1/CRE



**Figure 7.3 Map of protein binding sites in the 5'-flanking regions of the human CYP11B1 and CYP11B2 genes.** The regulatory DNA regions are shown by lines. Transcription factor binding sites are denoted by circles (SF1), squares (CRE) and oblongs (COUP-TF).

site in CYP11B2 genes studied in other species as well as bCYP11B. Thus, the expression of CYP11B1 appears to require similar *cis*-regulatory elements to those shown important for bCYP11B and mouse CYP11B2. The potential role of the AP-1 sites implicated in rCYP11B1 transcription has not been determined for CYP11B1 transcription.

The H295R cell line also has proven particularly useful in the analysis of human CYP11B2 because it retains angiotensin II and  $K^+$  signaling pathways that are not present in Y-1 cells (Clyne *et al.*, 1996). A reporter construct containing 2017 bp of CYP11B2 5' flanking DNA was stimulated by angiotensin II,  $K^+$ , BAYK8644 (an L-type  $Ca^{2+}$  channel agonist) and cAMP analogues, but was not affected by phorbol ester. Reporter constructs with serial deletions or site-directed mutations further localized functionally important elements, which then were characterized by electrophoretic mobility shift assays and DNase I footprinting analyzes. These experiments identified two elements at positions -78/-72 and -136/-121 that were both necessary and sufficient for basal reporter gene expression or for activity induced by either cAMP or calcium signaling pathways. The -78/-72 element

closely resembles a consensus cAMP response element (CRE) and corresponds to elements of known importance for expression of murine CYP11B2 and bCYP11B genes. The -136/-121 element has 12/15 bases matching the Ad5 element described in bCYP11B. Whereas mutation of the bovine Ad5 site has little effect on gene expression, mutation or deletion of this element in the human CYP11B2 reporter decreased cAMP, ANG II and K<sup>+</sup> stimulated expression. In electrophoretic mobility shift assays the -136/-121 element bound several H295R nuclear proteins. One complex corresponded to SF-1, whereas a second complex contained chicken ovalbumin upstream promoter transcription factor (COUP-TF), another orphan nuclear receptor (Wang *et al.*, 1989). Interestingly, co-transfection studies suggest that SF-1 inhibits expression of CYP11B2 (unpublished observation). This differs from studies with a CYP11B1 reporter in which activity was stimulated 10-fold by SF-1 co-expression. In summary, it is likely that calcium and cAMP signaling pathways share the same regulatory elements to control transcription of CYP11B2. Whereas further studies will be necessary to define which factors may be involved in the zone-specific expression of the two CYP11B isozymes, the different requirements for SF-1 may be a key difference in the regulation of transcription.

## STEROID 11 $\beta$ -HYDROXYLASE DEFICIENCY

### Clinical presentation

Congenital adrenal hyperplasia, the inherited inability to synthesize cortisol, can be caused by mutations in any of the latter four enzymes required for cortisol biosynthesis (see [Figure 6.1](#) in the previous chapter) or by mutations in the steroidogenic acute regulatory (StAR) protein required for cholesterol transport into mitochondria (Stocco and Clark, 1996b). More than 90% of cases are caused by 21-hydroxylase deficiency (see [Chapter 6](#)). This usually affects both aldosterone and cortisol biosynthesis, leading to signs of aldosterone deficiency including hyponatremia, hyperkalemia and hypovolemia that may, if untreated, progress to shock and death within weeks of birth. In contrast, most remaining cases of CAH are associated with hypertension and are due to 11 $\beta$ -hydroxylase deficiency (reviewed in White *et al.*, 1994). In most populations, 11 $\beta$ -hydroxylase deficiency comprises approximately 5–8% of cases of congenital adrenal hyperplasia (Zachmann *et al.*, 1983) and thus it occurs in approximately 1 in 200,000 births. A large number of cases of 11 $\beta$ -hydroxylase deficiency has been reported in Israel among Jewish immigrants from Morocco; the incidence in this group is currently estimated to be 1/5000–1/7000 births (Rosler *et al.*, 1992).

In 11 $\beta$ -hydroxylase deficiency, 11-deoxycortisol and deoxycorticosterone are not efficiently converted to cortisol and corticosterone, respectively. Decreased production of glucocorticoids reduces their feedback inhibition on the hypothalamus and anterior pituitary, increasing secretion of ACTH. This stimulates the zona fasciculata of the adrenal cortex to overproduce steroid precursors proximal to the blocked 11 $\beta$ -hydroxylase step. Thus, 11 $\beta$ -hydroxylase deficiency can be diagnosed by high basal or ACTH-stimulated levels of deoxycorticosterone and/or 11-deoxycortisol in the serum, or by increased excretion of the tetrahydro metabolites of these compounds in a 24 hour urine collection. Obligate

heterozygous carriers of 11 $\beta$ -hydroxylase deficiency alleles (e.g., parents) have no consistent biochemical abnormalities detectable even after stimulation of the adrenal cortex with intravenous ACTH (Pang *et al.*, 1980), consistent with an autosomal recessive mode of inheritance.

Approximately two-thirds of patients with the severe, "classic" form of 11 $\beta$ -hydroxylase deficiency have high blood pressure (Rosler *et al.*, 1992), often beginning in the first few years of life (Mimouni *et al.*, 1985). Although the hypertension is usually of mild to moderate severity, left ventricular hypertrophy and/or retinopathy have been observed in up to one-third of patients, and deaths from cerebrovascular accidents have been reported. Other signs of mineralocorticoid excess such as hypokalemia and muscle weakness or cramping occur in a minority of patients and are not well correlated with blood pressure. Plasma renin activity is usually suppressed in older children and levels of aldosterone are consequently low even though the ability to synthesize aldosterone is actually unimpaired.

The cause of hypertension in 11 $\beta$ -hydroxylase deficiency is not well understood. It might be assumed that it is caused by elevated serum levels of deoxycorticosterone but blood pressure and deoxycorticosterone levels are poorly correlated in patients. In addition, this steroid has only weak mineralocorticoid activity when administered to humans or other animals. Perhaps other metabolites of deoxycorticosterone are responsible for the development of hypertension. The 18-hydroxy and 19-nor metabolites of deoxycorticosterone are thought to be more potent mineralocorticoids (Griffing *et al.*, 1983), but consistent elevation of these steroids in 11 $\beta$ -hydroxylase deficiency has not been documented. Moreover, synthesis of these steroids requires hydroxylations within the adrenal (19-nor-deoxycorticosterone is synthesized via 19-hydroxy and 19-oic intermediates) that are probably mediated primarily by CYP11B1 (Ohta *et al.*, 1988). This is unlikely to take place efficiently in 11 $\beta$ -hydroxylase deficiency.

In addition to hypertension, patients with 11 $\beta$ -hydroxylase deficiency often exhibit signs of androgen excess. This occurs because accumulated cortisol precursors in the adrenal cortex are shunted (through the activity of 17 $\alpha$ -hydroxylase/17, 20-lyase) into the pathway of androgen biosynthesis, which is active in the human adrenal in both sexes. Affected females are born with some degree of masculinization of their external genitalia. This includes clitoromegaly and partial or complete fusion of the labioscrotal folds. Such ambiguous genitalia can be difficult to distinguish from those of a normal cryptorchid male (Harinarayan *et al.*, 1992; Rosler *et al.*, 1992; Bistritzer *et al.*, 1984). In contrast to the external genitalia, the gonads and the internal genital structures (Fallopian tubes, uterus and cervix) arising from the Mullerian ducts are normal and affected females have intact reproductive potential if their external genital abnormalities are corrected surgically.

Other signs of androgen excess that occur postnatally in both sexes include rapid somatic growth in childhood and accelerated skeletal maturation leading to premature closure of the epiphyses and short adult stature. Additionally, patients may have premature development of sexual and body hair (premature adrenarche) and acne. Androgens may affect the hypothalamic-pituitary-gonadal axis leading to amenorrhea or oligomenorrhea in females and true precocious puberty or, conversely, poor spermatogenesis in males (Hochberg *et al.*, 1985).

Glucocorticoid administration (usually hydrocortisone) replaces deficient cortisol and thus reduces ACTH secretion, suppressing excessive adrenal androgen production and preventing further virilization. Such therapy should also suppress ACTH-dependent production of mineralocorticoid agonists and ameliorate hypertension. If hypertension has been of long standing prior to treatment, additional anti-hypertensive drugs may be required to lower blood pressure into the normal range. These may include potassium sparing diuretics such as spironolactone or amiloride and/or a calcium channel blocker such as nifedipine (Nadler *et al.*, 1985). Because the renin-angiotensin system is suppressed in these patients, angiotensin converting enzyme inhibitors are unlikely to be effective. Thiazide diuretics should not be used except in combination with a potassium sparing diuretic because they will otherwise cause hypokalemia in patients with mineralocorticoid excess.

A mild, "nonclassic" form of 11 $\beta$ -hydroxylase has been described (Zachmann *et al.*, 1983) in which patients have relatively mild signs of androgen excess; they are not characteristically hypertensive. This disorder appears to be quite rare as compared with nonclassic 21-hydroxylase deficiency, and no mutations in CYP11B1 have been detected among women who are referred to reproductive endocrinology clinics for signs of androgen excess. It appears that ACTH-stimulated levels of 11-deoxycortisol are at least 5 times the upper limit of normal in patients with genuine 11 $\beta$ -hydroxylase deficiency (Johrer *et al.*, 1997).

### Genetic analysis

Deficiency of 11 $\beta$ -hydroxylase results from mutations in CYP11B1 (Figure 7.1). At this time, 20 mutations have been identified in patients with classic 11 $\beta$ -hydroxylase deficiency (Helmberg *et al.*, 1992; Curnow *et al.*, 1993; Naiki *et al.*, 1993; Geley *et al.*, 1996). In Moroccan Jews, a group that has a high prevalence of 11 $\beta$ -hydroxylase deficiency, almost all affected alleles carry the same mutation, Arg-448 to His (R448H) (White *et al.*, 1991). This probably represents a founder effect, but this mutation has also occurred independently in other ethnic groups, and another mutation of the same residue (R448C) has also been reported (Geley *et al.*, 1996). This apparent mutational "hotspot" contains a CpG dinucleotide. Such dinucleotides are prone to methylation of the cytosine followed by deamidation to TpG; several other mutations in CYP11B1 (T318M, R374Q, R384Q) are of this type.

These and almost all other missense mutations identified thus far are in regions of known functional importance (Poulos, 1991; Ravichandran *et al.*, 1993) and abolish enzymatic activity (Curnow *et al.*, 1993). For example, Arg-448 is adjacent to Cys-450 which is a ligand of the heme iron atom of this cytochrome P450 enzyme. T318M modifies an absolutely conserved residue that is thought to be critical for proton transfer to the bound oxygen molecule (Ravichandran *et al.*, 1993). E371G and R374Q also mutate highly conserved residues and may affect binding of adrenodoxin. R384Q is in a region that may form part of the substrate binding pocket (Ravichandran *et al.*, 1993). Almost all P450s have a basic residue (H or R) at this or the immediately adjacent position. Finally, V441G is adjacent to the highly conserved heme binding region, and this mutation may change the secondary structure of the protein.

Other mutations found in patients with the classic form of the disease are nonsense or frameshift mutations that also abolish enzymatic activity. One, a nonsense mutation of Trp-247 (W247X) has been identified in several unrelated kindreds in Austria and also probably represents a founder effect (Geley *et al.*, 1996).

Each patient with mild, nonclassic disease carries at least one mutation that reduces but does not destroy activity; the other mutation may be either mild or severe (Johrer *et al.*, 1997).

Although classic patients apparently completely lack 11 $\beta$ -hydroxylase activity, they differ significantly in the severity of the various signs and symptoms of their disease. There is no strong correlation between severity of hypertension and biochemical parameters such as plasma levels of the 11 $\beta$ -hydroxylase substrates, deoxycortisol and deoxycorticosterone, and urinary excretion of tetrahydro-deoxycortisol (White *et al.*, 1991; Rosler *et al.*, 1992). Moreover, there is no consistent correlation between the severity of hypertension and degree of virilization. These phenotypic variations must be governed by factors outside the CYP11B locus.

## ALDOSTERONE SYNTHASE (CORTICOSTERONE METHYLOXIDASE) DEFICIENCY

### Clinical presentation

By far the most frequent defect of aldosterone biosynthesis is congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency (see [Chapter 6](#)). Two-thirds of patients with classic 21-hydroxylase deficiency are unable to synthesize adequate amounts of aldosterone and are said to have the “salt-wasting” form of the disorder. Signs of androgen excess are prominent in such patients.

Rare patients have isolated aldosterone deficiency with entirely normal cortisol and sex steroid synthesis; this is caused by deficient activity of CYP11B2 (aldosterone synthase). The largest number of cases identified have been Iranian Jews from the city of Isfahan (Rosler, 1984), but the disease has been documented throughout Europe and North America (Veldhuis and Melby, 1981).

Mineralocorticoid deficiency leads to excessive sodium excretion and potassium retention in the renal distal tubule and cortical collecting duct, causing hyponatremia and hyperkalemia. In untreated infants with aldosterone synthase deficiency, serum sodium is usually in the range of 120–130mEq/l, whereas serum potassium ranges from 6.0 to 8.5. Children older than 3–4 years of age usually have normal serum electrolytes even if untreated. Plasma renin activity is markedly elevated (up to 100 times normal) in affected infants and young children, but it may be normal in adults.

Two forms of aldosterone synthase deficiency are recognized, termed corticosterone methyloxidase (i.e., aldosterone synthase) deficiency types I and II (Ulick, 1976). These syndromes have identical clinical features but differ in profiles of secreted steroids. Levels of deoxycorticosterone are increased and urinary excretion of corticosterone metabolites is elevated in both type I and type II deficiencies relative to excretion of cortisol metabolites. Whereas excretion of 18-hydroxycorticosterone is mildly decreased in type I deficiency,

urinary and serum levels of this steroid are dramatically increased in patients with type II deficiency. Aldosterone and its metabolites may be undetectable in patients with type I deficiency, whereas urinary excretion is mildly decreased in type II deficiency and serum levels of aldosterone are usually within normal limits. Type II deficiency may thus be readily diagnosed by marked (often 100-fold) elevation of the ratio of 18-hydroxycorticosterone to aldosterone in either urine or serum; the ratio does not vary with age in affected individuals and may be the sole biochemical abnormality in adults (Ulick *et al.*, 1992).

Levels of cortisol may be normal or elevated, and levels of adrenal androgens and their precursors are normal.

The clinical presentation of aldosterone synthase deficiency varies with age (Rosler, 1984; Ulick *et al.*, 1992). Infants may develop signs and symptoms of mineralocorticoid deficiency at a few days to weeks of age. These include vomiting and dehydration leading to hypovolemia that may cause cyanosis, tachycardia, hypotension, acidosis and prerenal azotemia. As discussed, hyponatremia and hyperkalemia are also characteristic of aldosterone deficiency. These problems may end in circulatory collapse. Although fatalities have occasionally occurred, the morbidity of aldosterone synthase deficiency is usually not as severe as that engendered by the salt-wasting form of congenital adrenal hyperplasia. This presumably reflects normal synthesis of deoxycorticosterone, corticosterone and cortisol in aldosterone synthase deficiency, which ameliorate the development of shock.

Some children are diagnosed in early childhood with failure to thrive, anorexia, mild dehydration and electrolyte abnormalities. Although electrolytes usually normalize by four years of age (even with a low sodium diet), growth retardation may persist throughout childhood. Adults are usually asymptomatic but occasionally tolerate severe salt loss (for example, from gastroenteritis) less well than unaffected individuals. Asymptomatic adults with type II deficiency are occasionally ascertained through family studies by the persistently elevated ratio of 18-hydroxycorticosterone to aldosterone (Rosler, 1984).

It is difficult to distinguish variations in clinical severity between individuals from the marked improvement that occurs with age in all patients. All affected individuals from the Iranian Jewish community have identical mutations (see below), so that any individual variations in severity that do exist cannot reflect allelic variation. They must instead represent effects of other genetic loci or non-genetic factors.

Although severely symptomatic infants require intravenous fluids, most infants and children are treated with oral sodium supplementation (2g/d as NaCl alone or in combination with NaHCO<sub>3</sub>) and fludrocortisone (0.1–0.3mg/d). Electrolyte abnormalities are quickly resolved but plasma renin activity and levels of steroid precursors may not return to normal for several months. In children in whom aldosterone synthase deficiency has resulted in failure to thrive, therapy may lead to dramatic catch-up growth.

Oral sodium supplements may be discontinued once plasma renin activity has decreased to normal but mineralocorticoid replacement therapy should be maintained throughout childhood until growth is complete. It is prudent to evaluate sodium balance upon discontinuing therapy.

### Genetic analysis

Mutations in CYP11B2 cause aldosterone synthase deficiency. Iranian Jewish patients with CMO II deficiency are all homozygous for two mutations, R181W and V386A. Thanks to an intragenic recombination segregating in these kindreds, it could be determined that homozygosity for either mutation alone was insufficient to cause disease. When these mutants were expressed in cultured cells, V386A alone had a minimal effect on activity, whereas R181W and the R181W/V386A double mutant both had intact 11 $\beta$ -hydroxylase activity, markedly decreased 18-hydroxylase activity and undetectable 18-oxidase activity. When expressed in a different cell line at higher levels of activity that were easier to quantify, the R181W, V386A and double mutants had 0.4%, 33% or 0.2% of wild type 18-oxidase activity, respectively. These findings suggest that 0.4% of normal 18-oxidase activity is not rate limiting for aldosterone biosynthesis, but 0.2% is. This is consistent with previous studies of congenital adrenal hyperplasia due to 21-hydroxylase deficiency; patients hemizygous for mutants with approximately 1% of wild type 21-hydroxylase activity are able to synthesize normal amounts of aldosterone.

It might be assumed that CMO I deficiency would be associated with null mutations in CYP11B2. Indeed one kindred with CMO I deficiency carried a frameshift mutation (Mitsuuchi *et al.*, 1993) and one carried a missense mutation, R384P, that destroyed enzymatic activity when expressed in cultured cells (Geley *et al.*, 1995). However, double homozygosity for mutations E198D and V386A is associated with CMO I deficiency although the double mutant enzyme retains 11 $\beta$ -hydroxylase activity in a manner similar to the R181W/V386A double mutant (Portrat-Doyen *et al.*, 1998).

Conversely, one patient with CMO II deficiency carried mutations on each chromosome that completely inactivated the enzyme; one was a frameshift and the other a missense mutation, T318M, affecting a residue crucial for catalysis. Although the frameshift and T318M should be completely sufficient to cause aldosterone synthase deficiency, each occurred in conjunction with one of the previously reported CMO II mutations, the frameshift on the same chromosome as R181W, and T318M on the same chromosome as V386A. The T318M/V386A double mutant was completely unable to synthesize aldosterone (Zhang *et al.*, 1995). Thus, CMO I and CMO II deficiencies are not simple allelic variants.

If the last patient carries mutations that completely inactivate the enzyme, why does he have CMO II and not CMO I deficiency? Evidently the high levels of 18-hydroxycorticosterone seen in CMO II deficiency (at least in this patient) are not being synthesized by CYP11B2. The 18-hydroxycorticosterone must be synthesized by CYP11B1 (which indeed does have some 18-hydroxylase activity) but then how can CMO I deficiency ever occur? Perhaps there is a polymorphism affecting expression of the 18-hydroxylase activity of CYP11B1; a polymorphism in the coding region might increase the actual 18-hydroxylase activity of the enzyme, whereas a polymorphism in a transcriptional control mechanism might increase CYP11B1 responsiveness to angiotensin II. If such a polymorphism exists, CMO II deficiency could occur only when a patient carried mutations on both CYP11B2 alleles that severely affected aldosterone synthase activity and also carried at least one "high 18-hydroxylase activity" CYP11B1 allele. Conversely, patients carrying two mutant CYP11B2 alleles and two "low 18-hydroxylase activity" CYP11B1 alleles would have a CMO I deficiency phenotype. Coding sequence polymorphisms of this nature have been



documented in the CYP11B1 gene in rats and have been associated with a salt-resistant phenotype as regards developing hypertension (Matsukawa *et al.*, 1993).

## GLUCOCORTICOID-SUPPRESSIBLE HYPERALDOSTERONISM

### Clinical presentation

Glucocorticoid suppressible hyperaldosteronism (also called dexamethasone-suppressible hyperaldosteronism or glucocorticoid-remediable aldosteronism) is a form of hypertension inherited in an autosomal dominant manner with high penetrance. It is characterized by moderate hypersecretion of aldosterone, suppressed plasma renin activity, and rapid reversal of these abnormalities after administration of glucocorticoids. It is clearly a rare disorder but until several years ago the absence of reliable biochemical or genetic markers made it difficult to ascertain.

Hypokalemia is usually mild and may be absent. Absolute levels of aldosterone secretion are usually moderately elevated in the untreated state but may be within normal limits. Plasma renin activity is strongly suppressed, so that the ratio of aldosterone secretion to renin activity is always abnormally high. Levels of 18-hydroxycortisol and 18-oxocortisol are elevated to 20–30 times normal. The ratio of urinary excretion of tetrahydro metabolites of 18-oxocortisol to those of aldosterone exceeds 2.0 whereas this ratio averages 0.2 in normal individuals (Rich *et al.*, 1992). Elevation of 18-oxocortisol is the most consistent and reliable biochemical marker of the disease, although it may also be elevated in cases of primary aldosteronism. This steroid may be of pathophysiologic significance; it is an agonist for the mineralocorticoid receptor and has been shown to raise blood pressure in animal studies (Hall and Gomez-Sanchez, 1986).

Once an affected individual has been identified in a kindred, additional cases may be ascertained within that kindred using biochemical (18-oxocortisol levels) (Rich *et al.*, 1992) or genetic (see below) markers. It is apparent from these studies that affected individuals have blood pressures that are markedly elevated compared to unaffected individuals in the same kindred, although some patients may in fact have normal blood pressures. Even young children typically have blood pressures greater than the 95th percentile for age, and most are frankly hypertensive before the age of 20. The hypertension is often of only moderate severity and blood pressures exceeding 180/120 are unusual. Associated signs of hypertension are frequent including left ventricular hypertrophy on the electrocardiogram and retinopathy. Some affected kindreds have remarkable histories of early (before age 45) death from strokes in many family members (Rich *et al.*, 1992).

Steroid biosynthesis is otherwise normal so that affected individuals have normal growth and sexual development.

Most laboratory and clinical abnormalities are suppressed by treatment with glucocorticoids, whereas infusion of ACTH exacerbates these problems (Oberfield *et al.*, 1981). This suggests that aldosterone is being inappropriately synthesized in the zona fasciculata and is being regulated by ACTH. Moreover, 18-hydroxycortisol and 18-oxocortisol, the steroids that are characteristically elevated in this disorder, are 17 $\alpha$ -hydroxylated analogs of 18-hydroxycorticosterone and aldosterone respectively. Because

17 $\alpha$ -hydroxylase is not expressed in the zona glomerulosa, the presence of large amounts of a 17 $\alpha$ -hydroxy-18-oxo-steroid suggests that an enzyme with 18-oxidase activity (i.e., aldosterone synthase, CYP11B2) is abnormally expressed in the zona fasciculata (White, 1991).

The initial treatment of choice in adults is dexamethasone (1–2mg/d). Children with this condition should be treated cautiously because of potential adverse effects of glucocorticoid therapy on growth. If therapy is indicated, children should be treated with the lowest effective dose of hydrocortisone. If hypertension is of long standing, it may not completely respond to glucocorticoids. This problem is similar to that observed in patients with 11 $\beta$ -hydroxylase deficiency and the choice of adjunctive therapy is governed by the same considerations. Patients with this disorder usually respond poorly to conventional antihypertensive medications unless they are also treated with glucocorticoids.

It is important to distinguish glucocorticoid suppressible hyperaldosteronism from aldosterone producing adenomas, considering that the latter condition is best treated by surgical removal of the affected adrenal gland (Melby, 1991). Secretion of 18-hydroxy- and 18-oxocortisol may be increased in patients with adenomas, but the ratio of urinary excretion of tetrahydro metabolites of 18-oxocortisol and aldosterone is rarely greater than 1.0. Suppression of aldosterone secretion with glucocorticoids and familial aggregation (Gordon *et al.*, 1992) are both unusual findings in adenomas but have been reported. However, presentation of an adenoma during childhood is exceedingly rare.

### Genetic analysis

All patients with glucocorticoid suppressible hyperaldosteronism have the same type of mutation, a chromosome that carries three CYP11B genes instead of the normal two (Figure 7.1) (lifton *et al.*, 1992a; Pascoe *et al.*, 1992a; Lifton *et al.*, 1992b). The middle gene on this chromosome is a chimera with 5' and 3' ends corresponding to CYP11B1 and CYP11B2 respectively. The chimeric gene is flanked by presumably normal CYP11B2 and CYP11B1 genes. In all kindreds analyzed thus far, the breakpoints (the points of transition between CYP11B1 and CYP11B2 sequences) are located between intron 2 and exon 4. As the breakpoints are not identical in different kindreds, these must represent independent mutations.

The chromosomes carrying chimeric genes are presumably generated by unequal crossing over. The high homology and proximity of the CYP11B1 and CYP11B2 genes makes it possible for them to become misaligned during meiosis. If this occurs, crossing over between the misaligned genes creates two chromosomes, one of which carries one CYP11B gene (i.e., a deletion) whereas the other carries three CYP11B genes.

The invariable presence of a chimeric gene in patients with this disorder suggests that this gene is regulated like CYP11B1 (expressed at high levels in the zona fasciculata and regulated primarily by ACTH) because it has transcriptional regulatory sequences identical to those of CYP11B1. If the chimeric gene has enzymatic activity similar to that of CYP11B2, a single copy of such an abnormally regulated gene should be sufficient to cause the disorder, consistent with the known autosomal dominant mode of inheritance of this syndrome. Recently, abnormal expression of the chimeric gene in the zona fasciculata was

directly demonstrated by *in situ* hybridization studies of an adrenal gland from a patient with this disorder (Pascoe *et al.*, 1995).

The chimeric genes causing glucocorticoid suppressible hyperaldosteronism may be readily detected by hybridization to Southern blots of genomic DNA, or they may be specifically amplified using the polymerase chain reaction (Jonsson *et al.*, 1995). As these techniques are widely used in molecular genetics laboratories, direct molecular genetic diagnosis may be more practical in many cases than assays of 18-oxocortisol levels, which are not routinely available (Dluhy and Lifton, 1995).

The limited region in which crossover breakpoints have been observed in glucocorticoid suppressible hyperaldosteronism alleles suggests that there are functional constraints on the structures of chimeric genes able to cause this disorder. One obvious constraint is that sufficient CYP11B2 coding sequences must be present in the chimeric gene so that the encoded enzyme actually has aldosterone synthase (i.e., 18-hydroxylase and 18-oxidase) activity. As determined by expressing chimeric cDNAs in cultured cells, chimeric enzymes with amino termini from CYP11B1 and carboxyl termini from CYP11B2 have 18-oxidase activity only if at least the region encoded by exons 5–9 corresponds to CYP11B2. If the sequence of exon 5 instead corresponds to CYP11B1, the enzyme has 11 $\beta$ -hydroxylase but no 18-oxidase activity (Pascoe *et al.*, 1992a). This is entirely consistent with the observation that no breakpoints in glucocorticoid-suppressible hyperaldosteronism alleles occur after exon 4. The chimeric enzymes either have strong 18-oxidase activity or none detectable and there does not appear to be any location of crossover that yields an enzyme with an intermediate level of 18-oxidase activity. Thus, there is no evidence for allelic variation in this disorder (i.e., variations in clinical severity are unlikely to be the result of different crossover locations).

Presumably the transcriptional regulatory region of the chimeric gene must correspond completely to that of CYP11B1 or the chimeric gene will not be expressed at sufficiently high levels in the zona fasciculata to cause the disorder. Although transcriptional regulatory elements in the CYP11B genes have not been completely defined, the fact that no breakpoints have been detected before intron 2 in glucocorticoid suppressible hyperaldosteronism alleles suggests that there is a transcriptional enhancer in exon 1-intron 2 of CYP11B1 or, conversely, a silencer in this region of CYP11B2.

Other factors such as kallikrein levels may affect the development of hypertension in this disorder (Dluhy and Lifton, 1995). One study found that blood pressure in persons with glucocorticoid-suppressible hyperaldosteronism is higher when the disease is inherited from the mother than when it is paternally inherited (Jamieson *et al.*, 1995). It is theoretically possible that the gene is imprinted (i.e., the maternal and paternal copies are expressed differently), but it seems more likely that exposure of the fetus to elevated levels of maternal aldosterone subsequently exacerbates the hypertension.

### Other forms of hyperaldosteronism

Most cases of primary hyperaldosteronism are sporadic. Frequencies of allelic variations in CYP11B2 (see the next section) are apparently not changed in patients with this disorder (Hampf *et al.*, 1998). Some kindreds exhibit an autosomal dominant form of

hyperaldosteronism that is not glucocorticoid-suppressible; in at least one such kindred, the disease is not linked to CYP11B2 (Torpy *et al.*, 1998). Obviously, additional loci must play a role in regulating CYP11B2 expression.

### Allelic variation in CYP11B2

Whereas it originally seemed possible that a "mild" form of glucocorticoid suppressible hyperaldosteronism might be a common etiology of essential hypertension, the lack of allelic variation in this disorder makes this unlikely. However, other polymorphisms in the 5' flanking region of CYP11B2 have been documented (Lifton *et al.*, 1992a; White and Slutsker, 1995), although none has been shown to affect expression of the gene. If any does influence regulation of CYP11B2, it might be a risk factor for the development of hypertension. It has also been suggested that polymorphisms in the coding sequence of CYP11B2 might increase the aldosterone synthase activity of the enzyme and thus might be a risk factor for hypertension (Fardella *et al.*, 1995). One coding sequence polymorphism, K173R, has been characterized in humans. It is not associated with any significant differences in enzymatic activity, but is associated with low renin hypertension in a small sample of patients from Chile.

Thus far, the most extensively studied polymorphism is located 344 nucleotides 5' of the start of translation; this position may be either a C or a T (−344C/T) (White and Slutsker, 1995). These alleles are present at approximately equal frequencies in Caucasian populations (White and Slutsker, 1995; Kupari *et al.*, 1998). This position comprises part of a binding site for the SF-1 transcription factor (see above), and the C allele binds SF-1 approximately four times as strongly as the T allele (unpublished observations). The functional significance of this is obscure, because this site may be deleted from reporter constructs without affecting expression (Clyne *et al.*, 1997). Inconsistent associations have been observed between this polymorphism and serum aldosterone levels or aldosterone excretion, with the C allele associated with higher aldosterone levels in some studies (Pojoga *et al.*, 1998), but lower levels in others (Brand *et al.*, 1998; Hautanen *et al.*, 1998a; Davies *et al.*, 1999). Associations with blood pressure have also been inconsistent (Pojoga *et al.*, 1998; Brand *et al.*, 1998; Hautanen *et al.*, 1998a; Kupari *et al.*, 1998; Davies *et al.*, 1999). Strong associations have been noted between −344C and other cardiovascular parameters including increased left ventricular diameter and mass, increased response of left ventricular mass to increases in dietary salt (Kupari *et al.*, 1998) and decreased baroreflex sensitivity (Ylitalo *et al.*, 1997). These associations all appear to be strongest in young adults and have been replicated in some but not other (Schunkert *et al.*, 1999) populations. Both left ventricular hypertrophy (Levy *et al.*, 1990) and decreased baroreflex sensitivity (La Rovere *et al.*, 1998) are well established predictors of morbidity and mortality from myocardial infarction, raising the possibility that the −344C/T polymorphism may represent an independent cardiovascular risk factor. Preliminary studies suggest that this may in fact be true, at least in high risk individuals with other risk factors such as dyslipidemias and smoking (Hautanen *et al.*, 1998b). At this time, it remains possible that the −344C/T polymorphism is merely a marker for an associated polymorphism that is affecting gene expression directly. To answer

this question, additional populations should be studied and the vicinity of CYP11B2 sequenced more completely in different individuals.

It is remarkable that the -344C/T polymorphism has such strong associations with left ventricular size, baroreflex sensitivity and (in one population) cardiovascular risk whereas associations with levels of aldosterone secretion are inconsistent. Thus one must consider an explanation for our observations other than differences in circulating aldosterone levels. For example, CYP11B2 expression has been reported in human (Takeda *et al.*, 1996) and rodent (Hatakeyama *et al.*, 1994) vascular endothelium and rodent heart (Silvestre *et al.*, 1998), so that aldosterone might have autocrine or paracrine effects on the heart and vasculature. If the -344C allele increased expression of CYP11B2 in these tissues, it might increase local concentrations of aldosterone and thus have cardiovascular effects without significantly increasing circulating aldosterone levels. Alternatively, another polymorphism in genetic linkage disequilibrium with -344C/T may be responsible for the observed associations. Elucidation of the mechanism (s) involved may provide useful insights into the role of aldosterone in the cardiovascular system.

### Mutation frequencies in CYP11B1 and CYP11B2

It is instructive to compare and contrast mutations causing 21-hydroxylase deficiency, the most common cause of congenital adrenal hyperplasia, with those observed in the CYP11B genes. Almost all 21-hydroxylase deficiency alleles result from recombinations between the normally active 21-hydroxylase gene, CYP21, and an adjacent pseudogene, CYP21P. These are either deletions of CYP21 due to unequal meiotic crossing-over or apparent gene conversions in which deleterious mutations normally present in CYP21P are transferred to CYP21 (Tusie-Luna and White, 1995).

Like CYP21 and CYP21P, CYP11B1 and CYP11B2 are closely linked homologs, but CYP11B1 and CYP11B2 both encode active enzymes. Thus, gene conversions that transfer polymorphic sequences between CYP11B1 and CYP11B2 are not expected to have major adverse effects on enzymatic activity, in which case genetic deficiencies of the encoded enzymes should result from mutations that are not gene conversions. This is the case for all CYP11B1 mutations causing 11 $\beta$ -hydroxylase deficiency that have been identified thus far, providing an explanation for the rarity of 11 $\beta$ -hydroxylase deficiency compared with 21-hydroxylase deficiency.

Whereas unequal crossovers resulting in a CYP11B1/2 duplication are associated with glucocorticoid-suppressible hyperaldosteronism, the reciprocal deleted chromosome should carry a single CYP11B gene with a 5' end corresponding to CYP11B2 and a 3' end corresponding to CYP11B1. Like CYP11B2, such a gene should be expressed at low levels and only in the zona glomerulosa, but it should encode an enzyme with activity similar to the normal product of CYP11B1. Such a chromosome might be expected to function as a null allele. As yet, no such chromosomes have been detected in patients with 11 $\beta$ -hydroxylase deficiency. This suggests that unequal crossovers of the CYP11B genes occur rarely compared with this type of rearrangement in the CYP21 genes. Similarly, no duplications of the CYP11B genes have been detected in more than 400 individuals in the general population (Lifton *et al.*, 1992b).

It is notable that many kindreds with glucocorticoid suppressible hyperaldosteronism are of Anglo-Irish extraction (Lifton *et al.*, 1992b; Pascoe *et al.*, 1992a). Moreover, the chromosomes carrying chimeric genes tend to occur in association with specific polymorphisms in the CYP11B genes (Lifton *et al.*, 1992b), even though the duplications generating the chimeric genes are apparently independent events. This suggests that one of these polymorphisms is, or is in linkage disequilibrium with, a structural polymorphism that predisposes to unequal crossing over during meiosis. Such features might include sequences similar to *chi* sites in bacteriophage lambda; this type of sequence has been postulated to increase the frequency of recombination in the CYP21 genes (Amor *et al.*, 1988). Additionally, in approximately 40% of alleles in Caucasians, the second intron of CYP11B2 has a sequence almost identical to that of CYP11B1 (White and Slutsker, 1995). This region could promote misalignment of chromosomal segments during meiosis and thus increase the risk of unequal crossing over.

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#### REFERENCES

- Adler, G.K., Chen, R., Menachery, A.I., Braley, L.M. and Williams, G.H. (1993) Sodium restriction increases aldosterone biosynthesis by increasing late pathway, but not early pathway, messenger ribonucleic acid levels and enzyme activity in normotensive rats. *Endocrinology* **133**, 2235–2240.
- Aguilera, G. (1993) Factors controlling steroid biosynthesis in the zona glomerulosa of the adrenal. [Review]. *J. Steroid Biochem. Mol. Biol.* **45**, 147–151.
- Allen, R.G., Carey, C., Parker, J.D., Mortrud, M.T., Mellon, S.H. and Low, M.J. (1995) Targeted ablation of pituitary pre-proopiomelanocortin cells by herpes simplex virus-1 thymidine kinase differentially regulates mRNAs encoding the adrenocorticotropin receptor and aldosterone synthase in the mouse adrenal gland. *Mol. Endocrinol.* **9**, 1005–1016.
- Amor, M., Parker, K.L., Globerman, H., New, M.I. and White, P.C. (1988) Mutation in the CYP21B gene (Ile-172-Asn) causes steroid 21-hydroxylase deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1600–1604.
- Bird, I.M., Hanley, N.A., Word, R.A., Mathis, J.M., McCarthy, J.L., Mason, J.I. and Rainey, W.E. (1993) Human NCI-H295 adrenocortical carcinoma cells: a model for angiotensin-II-responsive aldosterone secretion. *Endocrinology* **133**, 1555–1561.
- Bird, I.M., Mason, J.I. and Rainey, W.E. (1994) Regulation of type 1 angiotensin II receptor messenger ribonucleic acid expression in human adrenocortical carcinoma H295 cells. *Endocrinology* **134**, 2468–2474.
- Bird, I.M., Mathis, J.M., Mason, J.I. and Rainey, W.E. (1995) Ca(2+)-regulated expression of steroid hydroxylases in H295R human adrenocortical cells. *Endocrinology* **136**, 5677–5684.
- Bird, I.M., Pasquarette, M.M., Rainey, W.E. and Mason, J.I. (1996) Differential control of 17 $\alpha$ -hydroxylase and 3 $\beta$ -hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. *J. Clin. Endocrinol. Metab.* **81**, 2171–2178.

- Bistritzer, T., Sack, J., Eshkol, A., Zur, H. and Katznelson, D. (1984) Sex reassignment in a girl with 11 beta-hydroxylase deficiency. *Isr. J. Med. Sci.* **20**, 55–58.
- Boon, W.C., McDougall, J.G. and Coghlan, J.P. (1998) Hypothesis: aldosterone is synthesized by an alternative pathway during severe sodium depletion. "A new wine in an old bottle". *Clin. Exp. Pharmacol. Physiol.* **25**, 369–378.
- Bottner, B., Denner, K. and Bernhardt, R. (1998) Conferring aldosterone synthesis to human CYP11B1 by replacing key amino acid residues with CYP11B2-specific ones. *Eur. J. Biochem.* **252**, 458–466.
- Brand, E., Chatelain, N., Mulatero, P., Fery, I., Curnow, K.M., Jeunemaitre, X., Corvol, P., Pascoe, L. and Soubrier, F. (1998) Structural analysis and evaluation of the aldosterone synthase gene in hypertension. *Hypertension* **32**, 198–204.
- Cao, P.R. and Bernhardt, R. (1999) Interaction of CYP11B1 (cytochrome P-45011P) with CYP11A1 (cytochrome P-450sc) in COS-1 cells. *Eur. J. Biochem.* **262**, 720–726.
- Clyne, C.D., White, P.C. and Rainey, W.E. (1996) Calcium regulates human CYP11B2 transcription. *Endocr. Res.* **22**, 485–492.
- Clyne, C.D., Zhang, Y., Slutsker, L., Mathis, J.M., White, P.C. and Rainey, W.E. (1997) Angiotensin II and potassium regulate human CYP11B2 transcription through common *cis* elements. *Mol. Endocrinol.* **11**, 638–649.
- Curnow, K.M., Slutsker, L., Vitek, J., Cole, T., Speiser, P.W., New, M.I., White, P.C. and Pascoe, L. (1993) Mutations in the CYP11B1 gene causing congenital adrenal hyperplasia and hypertension cluster in exons 6, 7, and 8. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4552–4556.
- Curnow, K.M., Tusie-Luna, M.T., Pascoe, L., Natarajan, R., Gu, J.L., Nadler, J.L. and White, P.C. (1991) The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. *Mol. Endocrinol.* **5**, 1513–1522.
- Davies, E., Holloway, C.D., Ingram, M.C., Inglis, G.C., Friel, E.G., Morrison, C., Anderson, N.H., Fraser, R. and Connell, J.M. (1999) Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene CYP11B2. *Hypertension* **33**, 703–707.
- Denner, K., Rainey, W.E., Pezzi, V., Bird, I.M., Bernhardt, R. and Mathis, J.M. (1996) Differential regulation of 11 $\beta$ -hydroxylase and aldosterone synthase in human adrenocortical H295R cells. *Mol. Cell. Endocrinol.* **121**, 87–91.
- Dluhy, R.G. and Lifton, R.P. (1995) Glucocorticoid-remediable aldosteronism (GRA): diagnosis, variability of phenotype and regulation of potassium homeostasis. *Steroids* **60**, 48–51.
- Domalik, L.J., Chaplin, D.D., Kirkman, M.S., Wu, R.C., Liu, W.W., Howard, T.A., Seldin, M.F. and Parker, K.L. (1991) Different isozymes of mouse 11 $\beta$ -hydroxylase produce mineralocorticoids and glucocorticoids. *Mol. Endocrinol.* **5**, 1853–1861.
- Fardella, C.E., Rodriguez, H., Hum, D.W., Mellon, S.H. and Miller, W.L. (1995) Artificial mutations in P450c11AS (aldosterone synthase) can increase enzymatic activity: a model for low-renin hypertension? *J. Clin. Endocrinol. Metab.* **80**, 1040–1043.
- Geley, S., Johrer, K., Peter, M., Denner, K., Bernhardt, R., Sippell, W.G. and Kofler, R. (1995) Amino acid substitution R384P in aldosterone synthase causes corticosterone methyl oxidase type I deficiency. *J. Clin. Endocrinol. Metab.* **80**, 424–429.
- Geley, S., Kapelari, K., Johrer, K., Peter, M., Glatzl, J., Vierhapper, H., Sippell, W.G., White, P.C. and Kofler, R. (1996) CYP11B1 mutations causing congenital adrenal hyperplasia due to 11 $\beta$ -hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **81**, 2896–2901.
- Gordon, R.D., Klemm, S.A., Tunny, T.J. and Stowasser, M. (1992) Primary aldosteronism: hypertension with a genetic basis. *Lancet* **340**, 159–161.

- Griffing, G.T., Dale, S.L., Holbrook, M.M. and Melby, J.C. (1983) 19-nor-deoxycorticosterone excretion in primary aldosteronism and low renin hypertension. *J. Clin. Endocrinol. Metab.* **56**, 218–221.
- Hall, C.E. and Gomez-Sanchez, C.E. (1986) Hypertensive potency of 18-oxocortisol in the rat. *Hypertension* **8**, 317–322.
- Hall, P.F., Yanagibashi, K. and Kobayashi, Y. (1991) Synthesis of aldosterone by mitochondria and homogeneous 11 beta-hydroxylase from beef and pig. *Endocr. Res.* **17**, 135–149.
- Hampf, M., Widimsky, J. and Bernhardt, R. (1998) Aldosterone synthase gene in patients suffering from hyperaldosteronism. *Endocr. Res.* **24**, 877–880.
- Harinarayan, C.V., Ammini, A.C., Karmarkar, M.G., Prakash, V., Gupta, R., Taneja, N., Mohapatra, I., Kucheria, K. and Ahuja, M.M. (1992) Congenital adrenal hyperplasia and complete masculinization masquerading as sexual precocity and cryptorchidism. *Indian Pediatr.* **29**, 103–106.
- Hatakeyama, H., Miyamori, I., Fujita, T., Takeda, Y., Takeda, R. and Yamamoto, H. (1994) Vascular aldosterone. Biosynthesis and a link to angiotensin II-induced hypertrophy of vascular smooth muscle cells. *J. Biol. Chem.* **269**, 24316–24320.
- Hautanen, A., Lankinen, L., Kupari, M., Janne, O.A., Adlercreutz, H., Nikkila, H. and White, P.C. (1998a) Associations between aldosterone synthase gene polymorphism and the adrenocortical function in males. *J. Intern. Med.* **244**, 11–18.
- Hautanen, A., Toivanen, P., Manttari, M., Tenkanen, L., Manninen, V., Kayes, K.M., Rosenfeld, S. and White, P.C. (1998b) Variants of the aldosterone synthase gene and the risk of coronary heart disease in dyslipidemic middle-aged men. *Circulation* **98**, 1–531 (Abstract).
- Helmberg, A., Ausserer, B. and Kofler, R. (1992) Frame shift by insertion of 2 basepairs in codon 394 of CYP11B1 causes congenital adrenal hyperplasia due to steroid 11 $\beta$ -hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **75**, 1278–1281.
- Hochberg, Z., Schechter, J., Benderly, A., Leiberman, E. and Rosler, A. (1985) Growth and pubertal development in patients with congenital adrenal hyperplasia due to 11 $\beta$ -hydroxylase deficiency. *Am. J. Dis. Child.* **139**, 771–776.
- Honda, S., Morohashi, K., Nomura, M., Takeya, H., Kitajima, M. and Omura, T. (1993) Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *J. Biol. Chem.* **268**, 7494–7502.
- Jamieson, A., Slutsker, L., Inglis, G.C., Fraser, R., White, P.C. and Connell, J.M. (1995) Glucocorticoid-suppressible hyperaldosteronism: effects of crossover site and parental origin of chimaeric gene on phenotypic expression. *Clin. Sci.* **88**, 563–570.
- Johrer, K., Geley, S., Strasser-Wozak, E.M., Azziz, R., Wollmann, H.A., Schmitt, K., Kofler, R. and White, P.C. (1997) CYP11B1 mutations causing nonclassic adrenal hyperplasia due to 11 $\beta$ -hydroxylase deficiency. *Hum. Mol. Genet.* **6**, 1829–1834.
- Jonsson, J.R., Klemm, S.A., Tunny, T.J., Stowasser, M. and Gordon, R.D. (1995) A new genetic test for familial hyperaldosteronism type I aids in the detection of curable hypertension. *Biochem. Biophys. Res. Commun.* **207**, 565–571.
- Kakiki, M., Morohashi, K., Nomura, M., Omura, T. and Horie, T. (1997) Expression of aldosterone synthase cytochrome P450 (P450aldo) mRNA in rat adrenal glomerulosa cells by angiotensin II type 1 receptor. *Endocr. Res.* **23**, 277–295.
- Kawamoto, T., Mitsuuchi, Y., Ohnishi, T., Ichikawa, Y., Yokoyama, Y., Sumitomo, H., Toda, K., Miyahara, K., Kuribayashi, I., Nakao, K., Hosoda, K., Yamamoto, Y., Imura, H. and Shizuta, Y. (1990) Cloning and expression of a cDNA for human cytochrome P-450aldo as related to primary aldosteronism. *Biochem. Biophys. Res. Commun.* **173**, 309–316.



- Kawamoto, T., Mitsuuchi, Y., Toda, K., Yokoyama, Y., Miyahara, K., Miura, S., Ohnishi, T., Ichikawa, Y., Nakao, K., Imura, H., Ulick, S. and Shizuta, Y. (1992) Role of steroid 11 $\beta$ -hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1458–1462.
- Kupari, M., Hautanen, A., Lankinen, L., Koskinen, P., Virolainen, J., Nikkila, H. and White, P.C. (1998) Associations between human aldosterone synthase (CYP11B2) gene polymorphisms and left ventricular size, mass and function. *Circulation* **97**, 569–575.
- La Rovere, M.T., Bigger, J.T., Marcus, F.I., Mortara, A. and Schwartz, P.J. (1998) Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction. *Lancet* **351**, 478–484.
- Lala, D.S., Rice, D.A. and Parker, K.L. (1992) Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Mol. Endocrinol.* **6**, 1249–1258.
- Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B. and Castelli, W.P. (1990) Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study [see comments]. *N. Engl. J. Med.* **322**, 1561–1566.
- Lifton, R.P., Dluhy, R.G., Powers, M., Rich, G.M., Cook, S., Ulick, S. and Lalouel, J.M. (1992a) A chimaeric 11 $\beta$ -hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature* **355**, 262–265.
- Lifton, R.P., Dluhy, R.G., Powers, M., Rich, G.M., Gutkin, M., Fallo, F., Gill, J.R., Jr., Feld, L., Ganguly, A., Laidlaw, J.C., Murnaghan, D.J., Kaufman, C., Stockigt, J.R., Ulick, S. and Lalouel, J.M. (1992b) Hereditary hypertension caused by chimaeric gene duplications and ectopic expression of aldosterone synthase. *Nat. Genet.* **2**, 66–74.
- Matsukawa, N., Nonaka, Y., Higaki, J., Nagano, M., Mikami, H., Ogihara, T. and Okamoto, M. (1993) Dahl's salt-resistant normotensive rat has mutations in cytochrome P450(11 $\beta$ ) but the salt-sensitive hypertensive rat does not. *J. Biol. Chem.* **268**, 9117–9121.
- Melby, J.C. (1991) Diagnosis of hyperaldosteronism. *Endocrinol. Metab. Clin. North Am.* **20**, 247–255.
- Mellon, S.H., Bair, S.R. and Monis, H. (1995) P450c11B3 mRNA, transcribed from a third P450c11 gene, is expressed in a tissue-specific, developmentally, and hormonally regulated fashion in the rodent adrenal and encodes a protein with both 11-hydroxylase and 18-hydroxylase activities. *J. Biol. Chem.* **270**, 1643–1649.
- Mimouni, M., Kaufman, H., Roitman, A., Morag, C. and Sadan, N. (1985) Hypertension in a neonate with 11 $\beta$ -hydroxylase deficiency. *Eur. J. Pediatr.* **143**, 231–233.
- Mitani, F., Miyamoto, H., Mukai, K. and Ishimura, Y. (1996) Effects of long term stimulation of ACTH and angiotensin II-secrections on the rat adrenal cortex. *Endocr. Res.* **22**, 421–431.
- Mitsuuchi, Y., Kawamoto, T., Miyahara, K., Ulick, S., Morton, D.H., Naiki, Y., Kuribayashi, L., Toda, K., Hara, T., Orii, T., Yasuda, K., Miura, K., Yamamoto, Y., Imura, H. and Shizuta, Y. (1993) Congenitally defective aldosterone biosynthesis in humans: inactivation of the P450C18 gene (CYP11B2) due to nucleotide deletion in CMO I deficient patients. *Biochem. Biophys. Res. Commun.* **190**, 864–869.
- Mornet, E., Dupont, J., Vitek, A. and White, P.C. (1989) Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-45011 $\beta$ ). *J. Biol. Chem.* **264**, 20961–20967.
- Mountjoy, K.G., Robbins, L.S., Mortrud, M.T. and Cone, R.D. (1992) The cloning of a family of genes that encode the melanocortin receptors. *Science* **257**, 1248–1251.
- Mulatero, P., Curnow, K.M., Aupetit-Faisant, B., Fockling, M., Gomez-Sanchez, C., Veglio, F., Jeunemaitre, X., Corvol, P. and Pascoe, L. (1998) Recombinant CYP11B genes encode enzymes

- that can catalyze conversion of 11-deoxycortisol to cortisol, 18-hydroxycortisol, and 18-oxocortisol. *J. Clin. Endocrinol. Metab.* **83**, 3996–4001.
- Nadler, J.L., Hsueh, W. and Horton, R. (1985) Therapeutic effect of calcium channel blockade in primary aldosteronism. *J. Clin. Endocrinol. Metab.* **60**, 896–899.
- Naiki, Y., Kawamoto, T., Mitsuuchi, Y., Miyahara, K., Toda, K., Orii, T., Imura, H. and Shizuta, Y. (1993) A nonsense mutation (TGG [Trp 116]-TAG [Stop]) in GYP 11B1 causes steroid 11 $\beta$ -hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **77**, 1677–1682.
- Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K. and Nebert, D.W. (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12**, 1–51.
- Nelson, D.R. and Strobel, H.W. (1989) Secondary structure prediction of 52 membrane-bound cytochromes P450 shows a strong structural similarity to P450cam. *Biochemistry* **28**, 656–660.
- Nomura, M., Morohashi, K., Kirita, S., Nonaka, Y., Okamoto, M., Nawata, H. and Omura, T. (1993) Three forms of rat CYP11B genes: 11 $\beta$ -hydroxylase gene, aldosterone synthase gene, and a novel gene. *J. Biochem. (Tokyo)* **113**, 144–152.
- Oberfield, S.E., Levine, L.S., Stoner, E., Chow, D., Rauh, W., Greig, F., Lee, S.M., Lightner, E., Witte, M. and New, M.I. (1981) Adrenal glomerulosa function in patients with dexamethasone-suppressible hyperaldosteronism. *J. Clin. Endocrinol. Metab.* **53**, 158–164.
- Ogishima, T., Shibata, H., Shimada, H., Mitani, F., Suzuki, H., Saruta, T. and Ishimura, Y. (1991) Aldosterone synthase cytochrome P-450 expressed in the adrenals of patients with primary aldosteronism. *J. Biol. Chem.* **266**, 10731–10734.
- Ogishima, T., Suzuki, H., Hata, J., Mitani, F. and Ishimura, Y. (1992) Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-45011 $\beta$  in rat adrenal cortex: histochemical basis for the functional zonation. *Endocrinology* **130**, 2971–2977.
- Ohta, M., Fujii, S., Ohnishi, T. and Okamoto, M. (1988) Production of 19-oic-11-deoxycorticosterone from 19-oxo-11-deoxycorticosterone by cytochrome P-450(11 $\beta$ ) and nonenzymatic production of 19-nor-11-deoxycorticosterone from 19-oic-11-deoxycorticosterone. *J. Steroid Biochem.* **29**, 699–707.
- Pang, S., Levine, L.S., Lorenzen, F., Chow, D., Pollack, M., Dupont, B., Genel, M. and New, M.I. (1980) Hormonal studies in obligate heterozygotes and siblings of patients with 11 $\beta$ -hydroxylase deficiency congenital adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **50**, 586–589.
- Parker, K.L. and Schimmer, B.P. (1997) Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr. Rev.* **18**, 361–377.
- Pascoe, L., Curnow, K.M., Slutsker, L., Connell, J.M., Speiser, P.W., New, M.I. and White, P.C. (1992a) Glucocorticoid-suppressible hyperaldosteronism results from hybrid genes created by unequal crossovers between CYP11B1 and CYP11B2. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8327–8331.
- Pascoe, L., Curnow, K.M., Slutsker, L., Rosler, A. and White, P.C. (1992b) Mutations in the human CYP11B2 (aldosterone synthase) gene causing corticosterone methyl oxidase II deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4996–5000.
- Pascoe, L., Jeunemaitre, X., Lebrethon, M.C., Curnow, K.M., Gomez-Sanchez, C.E., Gasc, J.M., Saez, J.M. and Corvol, P. (1995) Glucocorticoid-suppressible hyperaldosteronism and adrenal tumors occurring in a single French pedigree. *J. Clin. Invest.* **96**, 2236–2246.
- Pezzi, V., Clyne, C.D., Ando, S., Mathis, J.M. and Rainey, W.E. (1997) Ca(2+)-regulated expression of aldosterone synthase is mediated by calmodulin and calmodulin-dependent protein kinases. *Endocrinology* **138**, 835–838.

- Picado-Leonard, J., Voutilainen, R., Kao, L.C., Chung, B.C., Strauss, J.F. and Miller, W.L. (1988) Human adrenodoxin: cloning of three cDNAs and cycloheximide enhancement in JEG-3 cells [published erratum appears in *J. Biol. Chem.* **263**, 11016, 1988]. *J. Biol. Chem.* **263**, 3240–3244.
- Pojoga, L., Gautier, S., Blanc, H., Guyene, T.T., Poirier, O., Cambien, F. and Benetos, A. (1998) Genetic determination of plasma aldosterone levels in essential hypertension. *Am. J. Hypertens.* **11**, 856–860.
- Portrat-Doyen, S., Tourniaire, J., Richard, O., Mulatero, P., Aupetit-Faisant, B., Curnow, K.M., Pascoe, L. and Morel, Y. (1998) Isolated aldosterone synthase deficiency caused by simultaneous E198D and V386A mutations in the CYP11B2 gene. *J. Clin. Endocrinol. Metab.* **83**, 4156–4161.
- Poulos, T.L. (1991) Modeling of mammalian P450s on basis of P450cam X-ray structure. *Methods Enzymol.* **206**, 11–30.
- Rainey, W.E., Rodgers, R.J. and Mason, J.I. (1992) The role of bovine lipoproteins in the regulation of steroidogenesis and HMG-CoA reductase in bovine adrenocortical cells. *Steroids* **57**, 167–173.
- Rainey, W.E. and White, P.C. (1998) Functional adrenal zonation and regulation of aldosterone biosynthesis. *Curr. Opin. Endocrinol. Diab.* **5**, 175–182.
- Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J. (1993) Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* **261**, 731–736.
- Rich, G.M., Ullick, S., Cook, S., Wang, J.Z., Lifton, R.P. and Dluhy, R.G. (1992) Glucocorticoid-remediable aldosteronism in a large kindred: clinical spectrum and diagnosis using a characteristic biochemical phenotype. *Ann. Intern. Med.* **116**, 813–820.
- Rosler, A. (1984) The natural history of salt-wasting disorders of adrenal and renal origin. *J. Clin. Endocrinol. Metab.* **59**, 689–700.
- Rosler, A., Leiberman, E. and Cohen, T. (1992) High frequency of congenital adrenal hyperplasia (classic 11 $\beta$ -hydroxylase deficiency) among Jews from Morocco. *Am. J. Med. Genet.* **42**, 827–834.
- Schunkert, H., Hengstenberg, C., Holmer, S.R., Broeckel, U., Luchner, A., Muscholl, M.W., Kurzinger, S., Doring, A., Hense, H.W. and Riegger, G.A. (1999) Lack of association between a polymorphism of the aldosterone synthase gene and left ventricular structure. *Circulation* **99**, 2255–2260.
- Shen, T., Suzuki, Y., Poyard, M., Best-Belpomme, M., Defer, N. and Hanoune, J. (1997) Localization and differential expression of adenylyl cyclase messenger ribonucleic acids in rat adrenal gland determined by *in situ* hybridization. *Endocrinology* **138**, 4591–4598.
- Silvestre, J.S., Robert, V., Heymes, C., Aupetit-Faisant, B., Mouas, C., Moalic, J.M., Swynghedauw, B. and Delcayre, C. (1998) Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. *J. Biol. Chem.* **273**, 4883–4891.
- Solish, S.B., Picado-Leonard, J., Morel, Y., R.W., Mohandas, T.K., Hanukoglu, I. and Miller, W.L. (1988) Human adrenodoxin reductase: two mRNAs encoded by a single gene on chromosome 17cen-q25 are expressed in steroidogenic tissues. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7104–7108.
- Stocco, D.M. and Clark, B.J. (1996a) Regulation of the acute production of steroids in steroidogenic cells. [Review]. *Endocr. Rev.* **17**, 221–244.
- Stocco, D.M. and Clark, B.J. (1996b) Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. [Review]. *Biochem. Pharmacol.* **51**, 197–205.
- Takeda, Y., Miyamori, I., Yoneda, M., Hatakeyama, H., Inaba, S., Furukawa, K., Mabuchi, H. and Takeda, R. (1996) Regulation of aldosterone synthase in human vascular endothelial cells by angiotensin II and adrenocorticotropin. *J. Clin. Endocrinol. Metab.* **81**, 2797–2800.

- Taymans, S.E., Pack, S., Pak, E., Torpy, D.J., Zhuang, Z. and Stratakis, C.A. (1998) Human CYP11B2 (aldosterone synthase) maps to chromosome 8q24.3. *J. Clin. Endocrinol. Metab.* **83**, 1033–1036.
- Torpy, D.J., Gordon, R.D., Lin, J.P., Huggard, P.R., Taymans, S.E., Stowasser, M., Chrousos, G.P. and Stratakis, C.A. (1998) Familial hyperaldosteronism type II: description of a large kindred and exclusion of the aldosterone synthase (CYP11B2) gene. *J. Clin. Endocrinol. Metab.* **83**, 3214–3218.
- Tusie-Luna, M.T. and White, P.C. (1995) Gene conversions and unequal crossovers between CYP21 (steroid 21-hydroxylase gene) and CYP21P involve different mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10796–10800.
- Ulick, S. (1976) Diagnosis and nomenclature of the disorders of the terminal portion of the aldosterone biosynthetic pathway. *J. Clin. Endocrinol. Metab.* **43**, 92–96.
- Ulick, S., Wang, J.Z. and Morton, D.H. (1992) The biochemical phenotypes of two inborn errors in the biosynthesis of aldosterone. *J. Clin. Endocrinol. Metab.* **74**, 1415–1420.
- Veldhuis, J.D. and Melby, J.C. (1981) Isolated aldosterone deficiency in man: acquired and inborn errors in the biosynthesis or action of aldosterone. *Endocr. Rev.* **2**, 495–517.
- Wada, A. and Waterman, M.R. (1992) Identification by site-directed mutagenesis of two lysine residues in cholesterol side chain cleavage cytochrome P450 that are essential for adrenodoxin binding. *J. Biol. Chem.* **267**, 22877–22882.
- Wagner, M.J., Ge, Y., Siciliano, M. and Wells, D.E. (1991) A hybrid cell mapping panel for regional localization of probes to human chromosome 8. *Genomics* **10**, 114–125.
- Wang, L.H., Tsai, S.Y., Cook, R.G., Beattie, W.G., Tsai, M.J. and O'Malley, B.W. (1989) COUP transcription factor is a member of the steroid receptor superfamily. *Nature* **340**, 163–166.
- Waterman, M.R. and Bischof, L.J. (1997) Cytochromes P450 12: diversity of ACTH (cAMP)-dependent transcription of bovine steroid hydroxylase genes. [Review]. *FASEB J.* **11**, 419–427.
- Waterman, M.R. and Simpson, E.R. (1989) Regulation of steroid hydroxylase gene expression is multifactorial in nature. *Recent Prog. Horm. Res.* **45**, 533–563.
- Weinstein, H. and Mehler, E.L. (1994) Ca(2+)-binding and structural dynamics in the functions of calmodulin. [Review] [140 refs]. *Annu. Rev. Physiol.* **56**, 213–236.
- White, P.C. (1991) Defects in cortisol metabolism causing low-renin hypertension. *Endocr. Res.* **17**, 85–107.
- White, P.C., Curnow, K.M. and Pascoe, L. (1994) Disorders of steroid 11 $\beta$ -hydroxylase isozymes. *Endocr. Rev.* **15**, 421–438.
- White, P.C., Dupont, J., New, M.I., Leiberman, E., Hochberg, Z. and Rosler, A. (1991) A mutation in CYP11B1 (Arg-448-His) associated with steroid 11 $\beta$ -hydroxylase deficiency in Jews of Moroccan origin. *J. Clin. Invest.* **87**, 1664–1667.
- White, P.C. and Slutsker, L. (1995) Haplotype analysis of CYP11B2. *Endocr. Res.* **21**, 437–442.
- Wong, M., Rice, D.A., Parker, K.L. and Schimmer, B.P. (1989) The roles of cAMP and cAMP-dependent protein kinase in the expression of cholesterol side chain cleavage and steroid 11 $\beta$ -hydroxylase genes in mouse adrenocortical tumor cells. *J. Biol. Chem.* **264**, 12867–12871.
- Yanagibashi, K., Haniu, M., Shively, J.E., Shen, W.H. and Hall, P. (1986) The synthesis of aldosterone by the adrenal cortex. Two zones (fasciculata and glomerulosa) possess one enzyme for 11 $\beta$ -, 18-hydroxylation, and aldehyde synthesis. *J. Biol. Chem.* **261**, 3556–3562.
- Ylitalo, A., Hautanen, A., Airaksinen, K.E.J., Savolainen, M.J., Kauma, H., Kupari, M., Kesaniemi, A. and Huikari, H. (1997) Baroreflex sensitivity and aldosterone synthase gene polymorphism in middle aged subjects. *Circulation* **96** (Suppl. 1), 227 (Abstract).

- Zachmann, M., Tassinari, D. and Prader, A. (1983) Clinical and biochemical variability of congenital adrenal hyperplasia due to 11 $\beta$ -hydroxylase deficiency. A study of 25 patients. *J. Clin. Endocrinol. Metab.* **56**, 222–229.
- Zhang, G., Rodriguez, H., Fardella, C.E., Harris, D.A. and Miller, W.L. (1995) Mutation T318M in the CYP11B2 gene encoding P450c11AS (aldosterone synthase) causes corticosterone methyl oxidase II deficiency. *Am. J. Hum. Genet.* **57**, 1037–1043.

8.  
**3 $\beta$ -HYDROXYSTEROID  
DEHYDROGENASE/  $\Delta^5$ - $\Delta^4$ -ISOMERASE  
DEFICIENCY**

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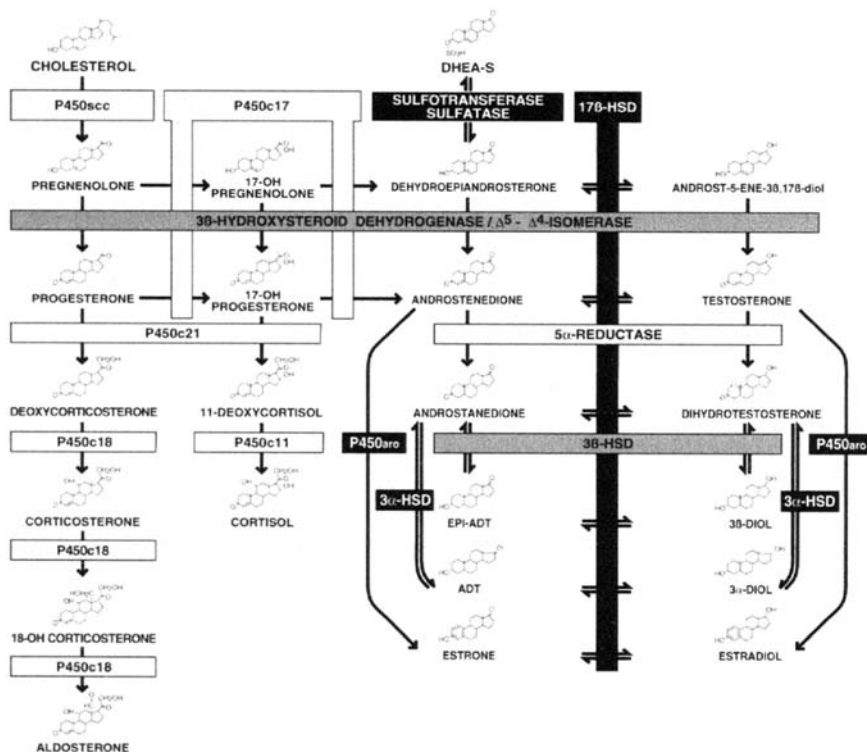
Classical 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase (3 $\beta$ -HSD) deficiency is a form of congenital adrenal hyperplasia that impairs steroidogenesis in both the adrenals and gonads resulting from mutations in the HSD3B2 gene and causing varying degrees of salt-wasting in both sexes and incomplete masculinization of the external genitalia in genetic males. Although an elevated ratio of  $\Delta^5$ / $\Delta^4$ -steroids was considered to be the best biological parameter for the diagnosis of this deficiency, the best criteria now appears to be plasma levels of 17-hydroxypregnenolone greater than 100nmol/L following stimulation with ACTH. To date, a total of 34 mutations (including five frameshift, four nonsense, one in-frame deletion, one splicing and 23 missense mutations) have been identified in the HSD3B2 gene in 56 individuals from 44 families suffering from classical 3 $\beta$ -HSD deficiency. The functional results obtained with mutant proteins are in agreement with the prediction that no functional type II 3 $\beta$ -HSD isoenzyme is expressed in the adrenals and gonads of the patients suffering from a severe salt-wasting form, whereas the nonsalt-losing form, also resulting from missense mutation(s) in the HSD3B2 gene, causes an incomplete loss in enzymatic activity, thereby leaving sufficient enzymatic activity to prevent salt wasting. Moreover, recent studies have highlighted the fact that various mutations appear to have a drastic effect upon the stability of the protein, therefore providing molecular evidence of a new mechanism involved in classical 3 $\beta$ -HSD deficiency. However, the functional data available concerning the sequence variants, which were detected in individuals with premature pubarche or hyperandrogenic adolescent girls suspected to be affected by non-classical 3 $\beta$ -HSD deficiency, coupled with the previous studies reporting that no mutations were found in both the HSD3B1 and/or HSD3B2 genes in such patients, strongly supports the conclusion that this disorder does not result from a mutant 3 $\beta$ -HSD isoenzyme. Finally, the functional characterization of missense mutations known to be involved in this autosomal recessive disorder associated with male pseudohermaphroditism also provides valuable information concerning the structure-function relationships of the 3 $\beta$ -HSD enzyme superfamily.

KEY WORDS: 3 $\beta$ -hydroxysteroid dehydrogenase deficiency, congenital adrenal hyperplasia, male pseudohermaphroditism, molecular diagnosis, steroidogenesis.

$3\beta$ -HYDROXYSTEROID DEHYDROGENASE/ $\Delta^5$ - $\Delta^4$ -ISOMERASE

## Biochemical characteristics

The NAD<sup>+</sup>-dependent membrane-bound enzyme  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase ( $3\beta$ -HSD), which resides in the endoplasmic reticulum and mitochondria (Luu-The *et al.*, 1989; Cherradi *et al.*, 1993, 1994, 1997; Sauer *et al.*, 1994; Thomas *et al.*,



**Figure 8.1** Schematic representation of the major mammalian steroidogenic pathways. P450scc: P450 cholesterol side-chain cleavage (CYP11A);  $3\beta$ -HSD,  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase; P450c21: P450 21 $\alpha$ -hydroxylase (CYP21); P450c17: P450 17 $\alpha$ -hydroxylase/17,20-lyase; P450c11 (CYP11B1): 11 $\beta$ -hydroxylase; P450c18 (CYP11B2): this enzyme mediates 11 $\beta$ -hydroxylation and the further reactions involved in the biosynthesis of aldosterone; 17 $\beta$ -HSD/KSR: 17 $\beta$ -hydroxysteroid dehydrogenase/17-keto-reductase; 5 $\alpha$ -reductases; P450aro: P450 aromatase (GYP 19).

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1998), catalyzes the sequential 3 $\beta$ -hydroxysteroid dehydrogenation and  $\Delta^5$  to  $\Delta^4$ -isomerization of the  $\Delta^5$ -steroid precursors pregnenolone (PREG), 17-hydroxypregnenolone (17OH-PREG), dehydroepiandrosterone (DHEA), and androst-5-ene-3 $\beta$ ,17 $\beta$ -diol ( $\Delta^5$ -diol) into their respective  $\Delta^4$ -keto-steroids, namely progesterone (PROG), 17 $\alpha$ -hydroxyprogesterone (17OH-PROG),  $\Delta^4$ -androstenedione ( $\Delta^4$ -DIONE) and testosterone (T). This bifunctional dimeric enzyme is therefore required for the biosynthesis of all classes of steroid hormones, namely glucocorticoids, mineralocorticoids, progesterone, androgens and estrogens (Figure 8.1). In addition, the enzymes of the 3 $\beta$ -HSD family also catalyze the formation and/or degradation of 5 $\alpha$ -androstanes and 5 $\alpha$ -pregnanes, such as 5 $\alpha$ -dihydrotestosterone (DHT) and 5 $\alpha$ -dihydroprogesterone (Simard *et al.*, 1996; Mason *et al.*, 1997; Payne *et al.*, 1997). The 3 $\beta$ -HSD isoenzyme is therefore recognized as the “gatekeeper” of steroidogenesis in the adrenal cortex, gonads, placenta and a variety of peripheral target tissues (Mason *et al.*, 1998). Transient expression of human 3 $\beta$ -HSD isoenzymes provided the first direct evidence that the 3 $\beta$ -HSD and  $\Delta^5$ - $\Delta^4$ -isomerase activities reside within a single protein (Lorence *et al.*, 1990a; Lachance *et al.*, 1990; Rheume *et al.*, 1991). However, data obtained from affinity alkylation (Thomas *et al.*, 1990) and inhibition experiments (Luu-The *et al.*, 1991) that suggested separate 3 $\beta$ -HSD and isomerase sites are also consistent with a bifunctional catalytic site adopting a different conformation for each activity, as suggested by tryptic peptides associated with both catalytic activities localized using affinity radiolabelled steroids (Thomas *et al.*, 1993, 1994, 1997). Additional studies have supported the hypothesis that NADH, the coenzyme product of the rate-limiting 3 $\beta$ -HSD reaction, induces a conformational change around the bound 3-oxo- $\Delta^5$ -steroid (the 3 $\beta$ -HSD product and the isomerase substrate) to activate the isomerase step (Thomas *et al.*, 1995). Finally, as revealed by site-directed mutagenesis of the human type I (placental) enzyme, His<sup>261</sup> appears to be a critical amino acid residue for 3 $\beta$ -HSD activity while Tyr<sup>253</sup> or Tyr<sup>254</sup> participate in the isomerase activity (Thomas *et al.*, 1998).

### Human 3 $\beta$ -HSD type I and type II genes and their related pseudogenes

The structure of the isoenzymes of the 3 $\beta$ -HSD family have been characterized during the past decade in the human and several other vertebrate species (Figure 8.2). Human type I 3 $\beta$ -HSD cDNA was isolated and characterized by Luu-The *et al.* following purification of 3 $\beta$ -HSD from human placenta (Luu-The *et al.*, 1988, 1989, 1990), and this sequence was later confirmed by other workers (Lorence *et al.*, 1990a; Nickson *et al.*, 1991). The second 3 $\beta$ -HSD enzyme, chronologically designated as type II, was isolated from a human adrenal cDNA library (Rheume *et al.*, 1991). The type I 3 $\beta$ -HSD gene (HSD3B1) encodes an enzyme of 372 amino acids with a  $K_m$  of <1 $\mu$ M, which is predominantly expressed in the placenta and peripheral tissues, such as the skin (principally in sebaceous glands), mammary gland, prostate and several others normal and tumoral tissues (Rheume *et al.*, 1991; Dumont *et al.*, 1992; Gingras and Simard, 1999; Gingras *et al.*, 1999). In comparison, the type II gene (HSD3B2), which encodes a protein of 371 a.a. with a  $K_m$  in the range of 1–4 $\mu$ M, shares 93.5% identity with the type I, and is almost exclusively expressed in the adrenals, the ovary and testis (Rheume *et al.*, 1991; Lachance *et al.*, 1991). The higher affinity of type I 3 $\beta$ -HSD



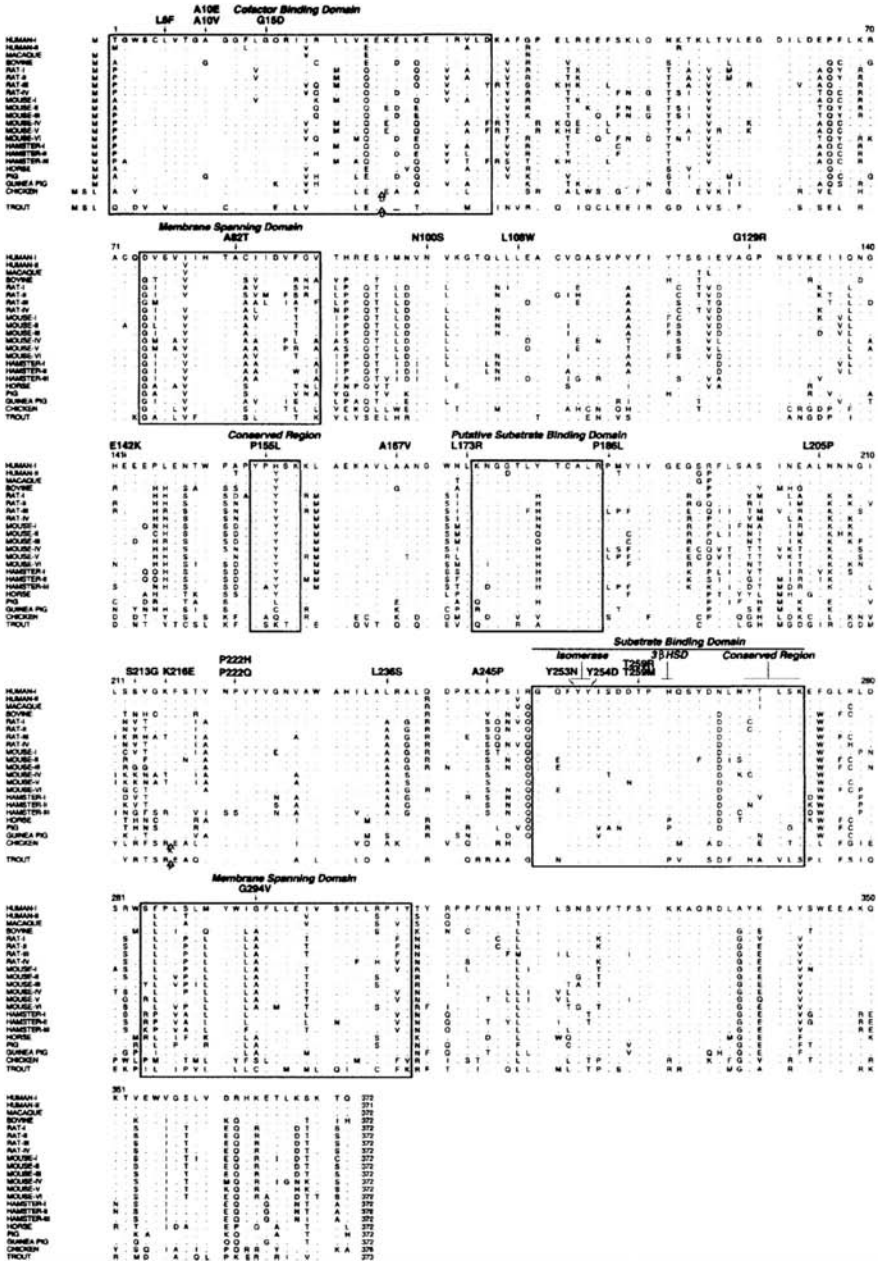
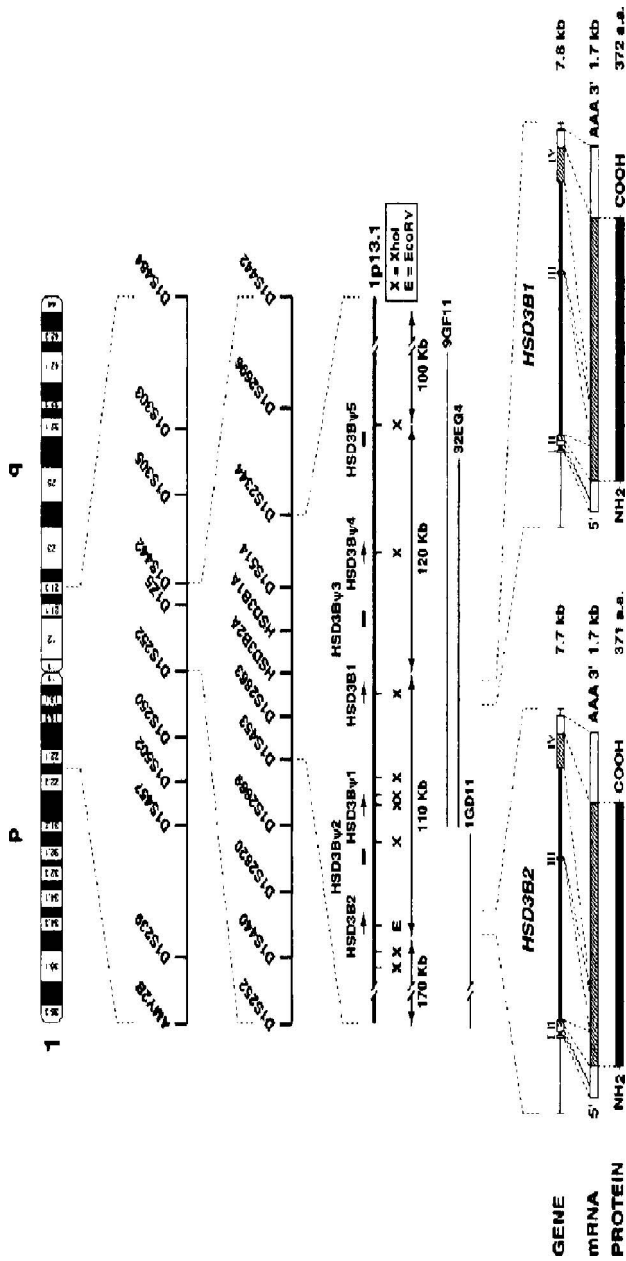


Figure 8.2 Comparison of the amino acid sequences of members of the 3β-HSD gene family: human types I and II; macaque ovary; bovine ovary; rat types I, II, III and IV; mouse types I, II, III, IV, V and VI; hamster types I, II and III; horse; pig; guinea-pig; chicken; and rainbow trout ovary. Residues common to the human type I 3β-HSD are represented by a dot. The members of the mammalian 3β-HSD family have been chronologically designated as a function of their elucidation in each species. The numbers indicated above refer to the human type II sequence. The missense mutations associated with 3β-HSD deficiency are shown with an arrow indicating their position in the human type II sequence.

could facilitate steroid formation from relatively low concentrations of substrates usually present in peripheral tissues. Based on their differential tissue-specific expression pattern, it is not surprising that classical 3 $\beta$ -HSD deficiency results from mutations in the HSD3B2 gene, whereas the HSD3B1 gene is normal in these affected individuals (Rheaume *et al.*, 1992; Simard *et al.*, 1993b, 1995). The structure of the HSD3B1 and HSD3B2 genes each consists of four exons included within a DNA fragment of 7.8kb, and sharing 77.4, 91.8, 94.5, 91.0% identity, respectively (Lachance *et al.*, 1990, 1991; Lorence *et al.*, 1990b). They were assigned to chromosome 1p13.1, 1–2cM from the centromeric marker D1Z5 (Figure 8.3) (Morissette *et al.*, 1995). Our initial data suggested that the HSD3B1 and HSD3B2 genes and three related pseudogenes (Luu-The *et al.*, 1992), are included within a 0.29 megabase *SacII* DNA fragment, suggesting that the human 3 $\beta$ -HSD gene family exists as a tandem cluster of related genes (Morissette *et al.*, 1995) as observed for the mouse 3 $\beta$ -HSD genes (Bain *et al.*, 1993). In support of these findings, in addition to the two expressed genes in the human, five pseudogenes have also been recently cloned and physically mapped (McBride *et al.*, 1999) (Figure 8.3). HSD3B $\psi$ 1-5 are unprocessed pseudogenes that are closely related to HSD3B1 and HSD3B2 genes but contain no corresponding open reading frames. Although mRNA is expressed from  $\psi$ 4 and  $\psi$ 5 in several tissues, altered splice sites disrupt the reading frames. The two expressed genes HSD3B1 and HSD3B2 are located in direct repeat, 100kb apart, but separation by two pseudogenes,  $\psi$ 1 and  $\psi$ 2, prevents them sharing common promoter elements (McBride *et al.*, 1999).

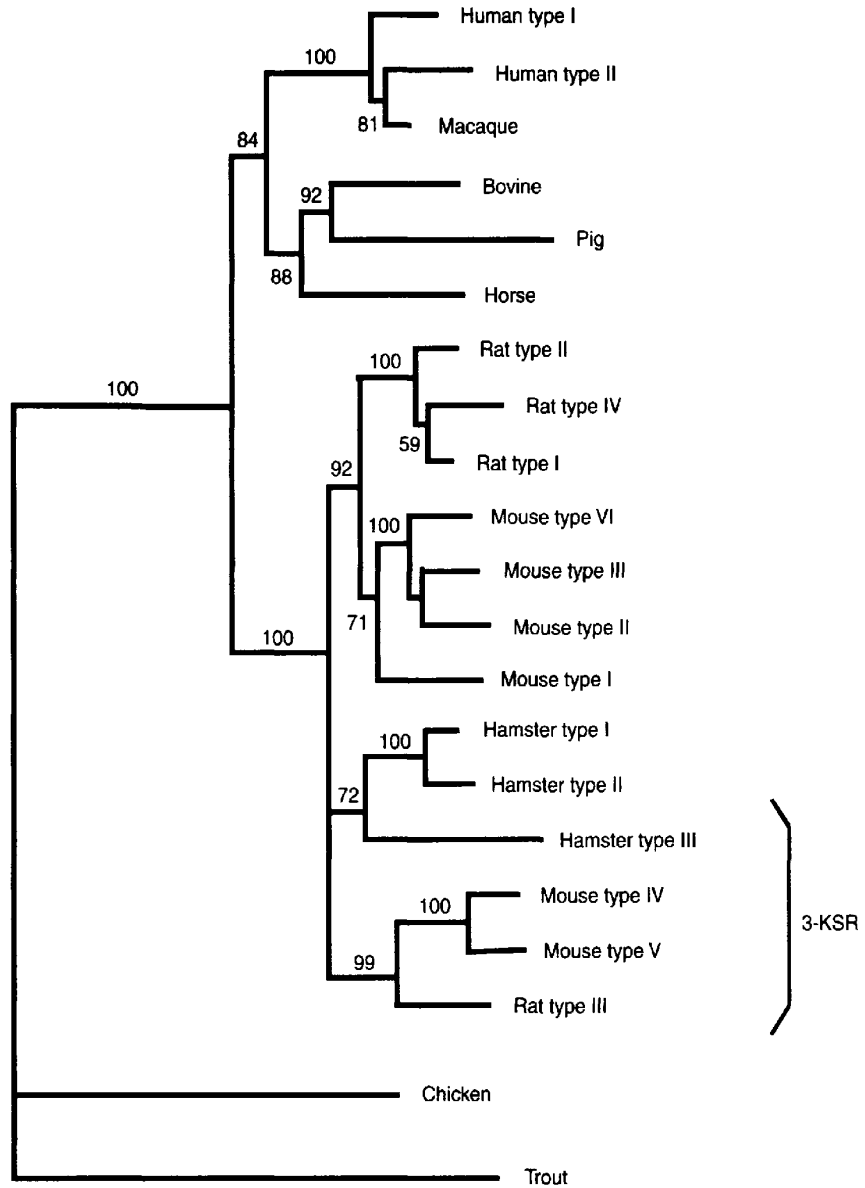
### Phylogeny of the vertebrate 3 $\beta$ -HSD gene family

Multiple 3 $\beta$ -HSD isoenzymes have also been cloned from several other species further illustrating that the 3 $\beta$ -HSD family is conserved in vertebrate species (Figure 8.2). The tissue-specific expression of multiple members of the 3 $\beta$ -HSD family was first demonstrated in the rat (Zhao *et al.*, 1990). Indeed, the structure of four types of rat 3 $\beta$ -HSD cDNAs, which all encode a 372 amino acid protein, has been elucidated (Zhao *et al.*, 1990, 1991; Lorence *et al.*, 1991; Simard *et al.*, 1993a), while six 3 $\beta$ -HSD isoenzymes have been characterized in the mouse (Bain *et al.*, 1991; Clarke *et al.*, 1993a, b; Keeney *et al.*, 1993; Abbaszade *et al.*, 1995; Abbaszade, 1997), and three isoenzymes have been cloned in the hamster (Rogerson *et al.*, 1996). Studies have also demonstrated that the structural organization of rat types I, II and IV 3 $\beta$ -HSD are closely related to the human 3 $\beta$ -HSD genes (Durocher *et al.*, 1993). Furthermore, 3 $\beta$ -HSD cDNAs have been cloned using adrenal/gonadal cDNA libraries from six other species, namely the macaque ovary (Simard *et al.*, 1991b), the bovine ovary (Zhao *et al.*, 1989), the chicken adrenal (Nakabayashi *et al.*, 1995), the horse testis (Hasegawa *et al.*, 1998), rainbow trout ovary (Sakai *et al.*, 1994), and the guinea-pig adrenal (Durocher F. and Simard J., personal communication). It is important to note that in contrast to the human, which is designated as type II, the adrenal/gonadal 3 $\beta$ -HSD isoenzymes in other vertebrate species have been designated as type I, due to the chronological order in which they were cloned. Finally, the only 3 $\beta$ -HSD sequence available from the pig was obtained using a cDNA library from adipose tissue (von Teichman *et al.*, unpublished article, Genbank accession



**Figure 8.3 Chromosomal localization showing the two expressed genes HSD3B1 and HSD3B2, and five pseudogenes,  $\psi$ 1–5.** The orientation of four genes is shown by the arrow that points towards the stop codon or its homologue. Clones of YACs (alpha numeric identification) are shown as a contig. The information regarding the order of the markets was obtained from the whitehead Institute/MIT center for Genome Research, Massachusetts, USA (<http://www.genome.wi.mit.edu/>). Structure of human type I and II  $\beta$ -HSD genes, mRNA species and the corresponding proteins. Exons are represented by boxes in which hatched lines demarcate the coding regions and open boxes represent the non-coding regions. Introns are represented by bold black lines.

number: AF232699). The male liver-specific rat type III protein (Zhao *et al.*, 1990; Couet *et al.*, 1992) does not display oxidative activity for the classical substrates, namely PREG, DHEA,  $\Delta^5$ -DIOL and 3P-DIOL, but instead is a specific 3-ketosteroid reductase (3-KSR), responsible for the conversion of 3-keto saturated steroids, such as DHT and 5 $\alpha$ -dihydroprogesterone, into inactive steroids using NADPH as the preferred cofactor instead of NAD<sup>+</sup> (H) (de Launoit *et al.*, 1992). Consequently, this liver-specific 3-KSR may be considered as an inactivating enzyme (Sanchez *et al.*, 1994a). The presence of a Tyr residue at position 36 in place of Asp in the typical  $\beta\alpha\beta$  dinucleotide-binding fold of the cofactor binding domain of rat type III is responsible for this difference in cofactor specificity of the rat 3-KSR (type III) protein, but it is not sufficient enough to explain its low activity with  $\Delta^5$ -3 $\beta$ -hydroxysteroid substrates (Simard *et al.*, 1996). The physiological importance of this peculiar member of the rat 3 $\beta$ -HSD family is well supported by the finding that mouse type IV, as well as male liver-specific mouse type V and hamster type III, also possess a specific 3-KSR activity (Abbaszade *et al.*, 1995; Rogerson *et al.*, 1998). The 3-KSR activity of the mouse type IV and type V as well as the hamster type III enzymes is most likely due to the presence of Phe36 instead of Asp36 (Figure 8.2). McBride *et al.* (1999) indicated that Southern blots revealed no evidence for the presence of other members of the human 3 $\beta$ -HSD family within the physical contig of 0.5Mb, thus suggesting that in the human there is no comparable liver-specific 3-KSR which could share a high % of identity with other members of the HSD3B cluster. Such a conclusion is also well supported by phylogenetic analysis of the mammalian 3 $\beta$ -HSD gene family. Unexpectedly, the phylogenetic tree obtained strongly suggests that independent gene duplications occurred in different species (Abbaszade *et al.*, 1995; McBride *et al.*, 1999; Vincent Laudet, personal communication) (Figure 8.4). As illustrated in Figure 8.4, the first complex of three genes from primates suggests that an ancestral gene duplicated specifically in the primate lineage to give rise to human types I and II, while the macaque gene is the homologue to human type II. It is very likely that a homologue of the human type I exists in the macaque genome. The second complex clusters together the single 3 $\beta$ -HSD species characterized in bovine, pig and horse. The third complex clusters together three clear clades of rodent 3 $\beta$ -HSD genes, firstly the rat type I, II and IV as well as the mouse type I, II, III and VI; secondly the mouse type IV, type V and rat type III, which are specific 3-KSRs; and thirdly the hamster type I, II and III. The hamster type III is a liver-specific 3-KSR (Rogerson *et al.*, 1998) and it is therefore surprising that it is not included in the second clade of rodent genes. These findings strongly suggest that the 3 $\beta$ -HSD genes were independently duplicated or triplicated three times in the lineage of the rat, the mouse and the hamster. It is not easy to understand why the duplication did not occur earlier in mammalian evolution if there are physiological needs and/or advantages for the presence of multiple isoenzymes. These data may indicate that the need for different 3 $\beta$ -HSD genes occurred very late in mammals, with subsequent evolution in a similar manner in other lineages. It is also of interest to note that although the N-terminal amino acid sequences of the pig hepatic 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase and the vertebrate 3 $\beta$ -HSD enzymes show some similarities, substrate specificities differ (Furster *et al.*, 1996; Furster, 1999). Furthermore, genetic studies of a kindred affected with 3 $\beta$ -hydroxy- $\Delta^5$ -C<sub>27</sub>-steroid dehydrogenase deficiency, which is associated with hepatic failure in childhood, showed no genetic linkage to the HSD3B cluster (Russell *et al.*, 1995). In fact such hepatic and extrahepatic activity was practically unaffected by trilostane, a well



**Figure 8.4** Unrooted phylogenetic tree constructed by the Neighbour-Joining method using 1000 bootstrap replicates (expressed as percentages). Nucleotide multiple alignments were obtained using PILEUP (Wisconsin GCG package) and the phylogenetic analysis was performed by PAUPsearch which provides a GCG interface to the tree-searching options in the PAUP program, version 4.0.0d55 (Phylogenetic Analysis Using Parsimony)(Swofford, 1993).

known C<sub>19</sub>/C<sub>21</sub> 3 $\beta$ -HSD inhibitor (Furster, 1999). Moreover, it has recently been suggested that the alcohol dehydrogenase  $\gamma$   $\gamma$  isoenzyme is the sole 3 $\beta$ -HSD using isobile acids as a substrate in human liver cytosol (Filling *et al.*, 1999). Finally, recently it has been demonstrated that the X-linked dominant, male-lethal phenotype gene mutated in bare patches and striated mice encodes a novel 3 $\beta$ -HSD (Liu *et al.*, 1999). This gene encodes an NAD(P)H enzyme which is likely to be involved in cholesterol biosynthesis and shares only 30% identity with other mammalian 3 $\beta$ -HSD enzymes, thus supporting the phylogenetic divergence between the C19/621 3 $\beta$ -HSD/ $\Delta^5$ - $\Delta^4$ -isomerase and the other enzymes involved in bile acid metabolism and/or biosynthesis of cholesterol.

## ONTOGENY AND CELL-SPECIFIC 3 $\beta$ -HSD GENE EXPRESSION

The 3 $\beta$ -HSD activity in steroidogenic tissues is an absolute requirement for mammalian reproduction, fetal growth and maintenance of life. Indeed, expression of 3 $\beta$ -HSD isoenzymes in placenta, fetal testis and the adrenal gland during human pregnancy is needed for the production of all classes of active steroid hormones. In order to better understand the consequences of an inherited defect in 3 $\beta$ -HSD activity we will briefly summarize, in the following pages, the knowledge concerning the ontogeny and cell-specific 3 $\beta$ -HSD gene expression.

### Localization and ontogeny in the human testis

In the human male fetus, Leydig cells can first be identified at approximately 8 weeks of gestation. They rapidly proliferate during the third month and the first half of the fourth month. The onset of T biosynthesis occurs at about 9 weeks of gestation (Siiteri, 1974). Human chorionic gonadotropin-luteinizing hormone (hCG-LH) receptors are present on fetal Leydig cells by at least week 12 of gestation, an observation suggesting that the initial secretion of T may be independent from hCG and LH (Grumbach and Conte, 1999). The Leydig cells secrete T which in turn promotes male differentiation of the Wolffian ducts, urogenital sinus and external genitalia. The number of Leydig cells decreases after 18 weeks and only a few cells continue to show Leydig cell characteristics in the interstitium of the testis at birth. The plasma concentration of T in the male fetus correlates with the biosynthetic activity of the fetal testis (Siiteri, 1974). Values comparable to those in the adult male (7 to 21 nmol/L) are found in the fetal circulation by about 16 weeks of gestation, while between 16 and 20 weeks the levels of T fall to about 3.5 nmol/L, and finally after week 24 the plasma level of T is low (comparable to that found at an early pubertal stage) (Grumbach and Conte, 1999). At the time of birth, plasma T levels are high during the first 24 hours and are very low shortly afterwards while they begin to rise after two weeks and remain elevated for approximately two to three months falling thereafter to low levels by the age of one year (Forest and Cathiard, 1975; Winter *et al.*, 1976; Bidlingmainer *et al.*, 1983). The T concentration remains low until the onset of puberty when the concentration again increases to reach adult levels by the age of 17. Human testicular interstitial cells were found to express 3 $\beta$ -HSD activity maximally before any other tissues, except for the trophoblast, as evaluated by histochemistry (Goldman *et al.*, 1966). A marked increase in 3 $\beta$ -HSD activity occurs at

the time of embryonic development when the testis has the highest concentrations of Leydig cells. At 8 weeks of gestation, George and Wilson noted the capacity of the fetal testis to synthesize T when incubated with  $C_{21}$ -steroid precursors, in contrast to the fetal adrenal gland (George and Wilson, 1979). Moreover, Baillie *et al.* (1965) were able to detect  $3\beta$ -HSD activity in the testes of human fetuses from 8 to 22 weeks of gestation by enzyme histochemistry. Furthermore, it was observed that by using second trimester fetal tissues, the specific  $3\beta$ -HSD activity in fetal testicular tissue (type II) was similar to that of the placental enzyme (type I), and that the activity was significantly higher than in other tissues including the adrenal neocortex, liver, intestine, kidney, lung, ovary, brain, skin, spleen and pancreas (Milewich *et al.*, 1991).  $3\beta$ -HSD immunostaining was reported in Leydig cells between 18–19 weeks of gestation (Parker *et al.*, 1995). In the testis of 22 week-old fetuses, immunostaining was observed exclusively in the cytoplasm of interstitial cells, whereas the seminiferous tubular elements remained completely unreactive (Dupont *et al.*, 1991). At 28 to 31 weeks of fetal life, strong immunostaining could be detected in the cytoplasm of interstitial cells. In 8 month-old infants and during childhood, no significant immunostaining for  $3\beta$ -HSD was observed in the testis. As observed in sections through the testis from a 15 year-old boy, the seminiferous tubules appeared to be well developed and the interstitial cells were immunoreactive, with the same intensity of immunostaining also being observed in adult testes (Dupont *et al.*, 1991). In summary,  $3\beta$ -HSD can be detected in Leydig cells in the fetus while immunostaining is very weak from 8 months to 12 years of age with increasing expression detectable at puberty.

### Localization and ontogeny in the human ovary

The ovaries, in contrast to the testes, remain relatively quiescent throughout fetal life and childhood. Moreover, the human fetal ovary does not contribute significantly to the levels of circulating estrogens, which in the fetus are almost exclusively of placental origin. The ovary has no documented role in differentiation of the female genital tract since development of the uterus and tubes occurs irrespective of whether the gonad is present or not (Grumbach and Conte, 1999). During human fetal life, immunostaining for  $3\beta$ -HSD in the ovary can be detected in theca interna cells surrounding the primary follicles and also in interstitial cells (Dupont *et al.*, 1992). Immunostaining was first observed at 28 weeks of gestation and persisted throughout the third trimester of gestation. At 34 weeks of fetal life, staining was observed not only in thecal cells of growing follicles, but also in the interstitial cells. Such results suggest that human fetal ovaries may be involved in sex steroid synthesis during the last weeks of gestation, especially as we know that other steroid-synthesizing enzymes, such as aromatase, are also expressed in the fetal ovary. This finding was rather unexpected since previous reports have clearly indicated that only minimal amounts of estrogens can be synthesized by fetal ovaries in several mammalian species, including man. The high levels of estrogens found in the fetal circulation are in fact thought to originate from the placenta (Kaplan and Grumbach, 1978). Finally the development of primordial follicles to the stage of primary follicles as well as the expression of  $3\beta$ -HSD observed from the 28th week of gestation could be related to the increase in fetal plasma FSH and LH levels, which occurs during the end of the second trimester of gestation (Kaplan and Grumbach, 1978;

Grumbach and Conte, 1999). Following parturition until puberty no immunostaining could be detected in any ovarian structure. During this period, only primordial and small primary follicles were observed, which is in agreement with the low levels of sex steroids observed during this period of life (Feinberg, 1965). From puberty until menopause, immunostaining was seen in theca interna cells in large primary as well as secondary and mature follicles. Atretic follicles often exhibited immunostaining of the theca interna, while primordial and small primary follicles were devoid of any immunoreaction. The theca externa did not show any labeling during folliculogenesis. Granulosa cells did not express 3 $\beta$ -HSD immunoreactivity in primary and secondary follicles, while in large antral and mature follicles, these cells exhibited labeling which was always less intense than that observed in thecal cells. Interestingly, one to several layers of the theca interna cells lying directly beneath the basement membrane did not show any immunoreactivity (Sasano *et al.*, 1990). In the corpus luteum, immunostaining was detected in both luteinized theca and granulosa cells, with equal intensity in both cell types (Dupont *et al.*, 1992), consistent with previous histochemical reports (Feinberg, 1965; Jones, 1968) and immunohistochemical data (Sasano *et al.*, 1990). In the menstrual and proliferative phase, expression of CYP11A, 3 $\beta$ -HSD and CYP17 became discernible in large-sized pre-antral follicles, i.e., more than five layers of granulosa cells, prior to the expression of CYP19, suggesting that not only estrogens but also PROG and androgens were essential for follicular development (Suzuki *et al.*, 1993, 1994). Indeed, expression of 3 $\beta$ -HSD is low and CYP17 is high during the follicular phase when estrogen synthesis is maximal in the human (Doody *et al.*, 1990a). It was therefore postulated that a higher ratio of CYP17/ 3 $\beta$ -HSD in the primate follicle favors  $\Delta^5$  metabolism and synthesis of androgens, and thereby estrogens, before ovulation, whereas a lower ratio favors PROG synthesis in the corpus luteum (Conley and Bird, 1997). It is also of interest to note that is likely that a reduction of a component in the steroidogenic pathway, particularly 3 $\beta$ -HSD, is involved in the continued fall in PROG production premenstrually (Duncan *et al.*, 1999). However, in the presence of logarithmically increasing concentrations of hCG in early pregnancy, the steroidogenic pathway is maintained, facilitating the continuing luteal synthesis of PROG. In menopausal and postmenopausal women, 3 $\beta$ -HSD immunoreactivity was found in dispersed interstitial cells, thus suggesting that some steroidogenesis could occur after the menopause (Dupont *et al.*, 1992). However, the main steroid secreted by human ovary after menopause is  $\Delta^4$ -DIONE. In fact, in postmenopausal women, about 30% of circulating  $\Delta^4$ -DIONE is of ovarian origin, the remaining being from adrenal sources (Vermeulen, 1976; Labire *et al.*, 1997; Labire, 1997).

### Localization and ontogeny in human placenta

The production of progesterone in the placenta catalyzed by type I 3 $\beta$ -HSD activity is essential for the maintenance of human pregnancy, especially following the decline in maternal ovarian progesterone synthesis, which occurs between the 8th and 10th week of pregnancy. During human pregnancy progesterone synthesis occurs predominantly in the placenta but also in the chorion and decidua. Consequently, it is not surprising that to this date no mutation has been reported in the HSD3B1 gene. It has generally been considered that the multinucleated syncytiotrophoblastic cell mass is the principal site of type I 3 $\beta$ -HSD



expression and, moreover, that 3 $\beta$ -HSD expression is intimately associated with cyclic AMP-promoted formation of syncytia (Mason *et al.*, 1993, 1997). This is in close agreement with the observation that the predominant sites of 3 $\beta$ -HSD expression, as revealed by immunocytochemistry throughout gestation, are principally the syncytiotrophoblast and the intermediate trophoblast cells in the placenta and the trophoblast cells in the chorion and decidua in fetal membranes (Riley *et al.*, 1992; Hawes *et al.*, 1994). The presence of 3 $\beta$ -HSD immunoreactivity and mRNA species was observed in uninucleate cytotrophoblasts in the chorion laeve, similar to that seen in syncytia, but they were notable by their absence in cytotrophoblast cells of the placenta (Mason *et al.*, 1993). Moreover, there was no change in type I 3 $\beta$ -HSD mRNA levels in either the placenta or chorio-decidua obtained after cesarean section at term, after preterm labor, or after term or post-term vaginal delivery, thus suggesting that the expression of this gene and decreased progesterone production are unlikely to affect intrauterine regulatory mechanisms leading to term or preterm labor in women (Riley *et al.*, 1993).

### Localization and ontogeny in the human adrenal gland

Circulating levels of free and conjugated 3 $\beta$ -hydroxy- $\Delta^5$ -steroids undergo dramatic changes with a rapid decline after birth, a gradual rise during late childhood and puberty, and a smaller decline in old age (de Peretti *et al.*, 1978; Dickerman *et al.*, 1984; Labrie *et al.*, 1997). Although DHEA and its sulfate are inactive steroid precursors, they are important substrates for placental estrogen synthesis (Grumbach and Conte, 1999), and formation of active androgens and estrogens in peripheral tissues (Labrie, 1991, 1997; Labrie *et al.*, 1999). Analyses of intraadrenal, circulating and urinary  $\Delta^4$ - to  $\Delta^5$ -steroid ratios during development have suggested that these phenomena reflect, at least in part, changes in the activity of the adrenal type II 3 $\beta$ -HSD complex. The fetal adrenal gland derives from the dorsal coelomic epithelium, and at 8 weeks of gestation, the human adrenal comprises chromaffin cells which will later form the adrenal medulla as well as the cortex. The human fetal adrenal cortex is morphologically and functionally differentiated into two zones, namely the outer or neocortex and the inner or fetal zone. The fetal zone primarily secretes  $\Delta^5$ -steroid 3-sulfates, whereas the neo-cortex secretes higher amounts of 3-keto- $\Delta^4$ -steroids and is the primary site of cortisol production in the fetus (Seron-Ferre, 1978; Simonian and Gill, 1981). The neo-cortex further differentiates into the zona glomerulosa, fasciculata and reticularis during the period of infancy and early childhood, while the inner zone, which occupies approximately 80% of the adrenal volume during fetal life, regresses following birth. Alternatively, it has also been suggested that the adrenal cortex in the fetus of both the human and the rhesus monkey is comprised of three, rather than two functional zones; the definitive zone, which may be analogous to the nascent zona glomerulosa, the transitional zone which is analogous to the zona fasciculata, and the fetal zone which is analogous to the zona reticularis, which respectively produce mineralocorticoids, glucocorticoids and the C<sub>19</sub> steroids DHEA and DHEA-S (Mesiano *et al.*, 1993). In early gestation, 11–15 weeks, considerable 3 $\beta$ -HSD was observed in the neocortex and in occasional fetal zone cells (Parker *et al.*, 1995). Thereafter, until 24–25 weeks, 3 $\beta$ -HSD

expression was very low in neocortex cells and virtually absent from the fetal zone (Parker *et al.*, 1995). Indeed, in the fetal adrenal at 22 weeks of gestation 3 $\beta$ -HSD immunostaining was observed exclusively in the cytoplasm of cells of the neo-cortex (Dupont *et al.*, 1990). It was also reported that 3 $\beta$ -HSD protein and mRNA levels were absent from the human fetal gland at mid-gestation (Doody *et al.*, 1990b; Voutilainen *et al.*, 1991; Mesiano *et al.*, 1993). Throughout the third trimester, the outer 1/2–2/3 of the neo-cortex was increasingly immunostained and clusters of immunoreactive cells also appeared near the central medullary vein of the adrenal (Dupont *et al.*, 1990; Parker *et al.*, 1995). The neocortex cells and those located in the cortical cuff region that expressed 3 $\beta$ -HSD resembled zona glomerulosa cells, while strong staining was observed in the presumptive zona glomerulosa in term fetal adrenals (Dupont *et al.*, 1990; Parker *et al.*, 1995). These results are consistent with the presence of a hitherto unknown stimulus for 3 $\beta$ -HSD in the human fetal adrenal in early gestation, followed by a suppression of the adrenal concentration of this enzyme during mid-gestation. High expression of 3 $\beta$ -HSD in early development may facilitate cortisol production, independently from placental transfer of cortisol, which is believed to play a role in the differentiation of medullary precursors during this developmental period (Parker *et al.*, 1995). The control of adrenal type II 3 $\beta$ -HSD gene expression during human fetal development may be more complex than initially envisioned and requires further analysis. It was suggested that the increase in fetal pituitary ACTH secretion plays an important role in the induction of type II 3 $\beta$ -HSD expression in the transitional zone of the primate fetal adrenal (Coulter *et al.*, 1996; Albrecht *et al.*, 1996). In 2-month-old infant adrenals, immunostaining was observed in both the zona glomerulosa and the zona fasciculata, while no staining could be detected in the easily identified zona reticularis (Dupont *et al.*, 1990). Similar 3 $\beta$ -HSD localization was also seen in 8-month-old infant adrenals, while in 2-year-old infants and adults, 3 $\beta$ -HSD immunostaining was observed in the three layers of the cortex, the intensity of staining being similar in the zona reticularis, fasciculata and glomerulosa, while the medulla was devoid of any immunoreactivity (Dupont *et al.*, 1990). Adrenarche, defined as an increased production in C<sub>19</sub> steroids (DHEA and DHEA-S), occurs between 6 and 8 years of age, but is not accompanied by an increase in cortisol secretion. In most cases this precedes the development of pubic hair, which occurs at a mean age of 10.5 years in Caucasian girls and 8.8 years in African-American girls (Herman-Giddens, 1997). This phenomena is limited to humans and chimpanzees (Cutler, 1978) and although the mechanism is not well understood, it is known to be independent of the gonadotrophin-releasing hormone pulse generator activation that occurs at sexual maturation. It has recently been reported that as children mature there is a decrease in the expression of 3 $\beta$ -HSD in the adrenal reticularis that may contribute to the increased production of DHEA and DHEA-S seen during adrenarche (Gell *et al.*, 1998; Dardis *et al.*, 1999). Adrenal sections of children less than 5 years of age demonstrated greater 3 $\beta$ -HSD immunostaining in the zona reticularis than in the reticularis of children aged 8–13 years (Gell *et al.*, 1998). This decline, which was also observed for type II 3 $\beta$ -HSD mRNA levels (Dardis *et al.*, 1999), may play a key role in the increase in adrenal DHEA and DHEA-S secretion. Alternatively, it was proposed that adrenarche may be associated with the age-dependent growth of a 3 $\beta$ -HSD-deficient adrenal reticularis. However, because the level of 3 $\beta$ -HSD relative to CYP17 influences  $\Delta^4$ -steroid production,

it was also suggested that modulations in the ratio of 17 $\alpha$ -hydroxylase/17, 20 lyase activities catalysed by the CYP17 may be involved in the regulation of DHEA and DHEA-S synthesis, particularly at adrenarche (Conley and Bird, 1997; Miller, 1997; Mapes *et al.*, 1999). Moreover, it has been suggested that regulation of cytochrome b<sub>5</sub> expression plays an important role in the promotion of efficient 17,20 lyase activity and then androgen synthesis by the CYP17/reductase complex (Mapes *et al.*, 1999 and references therein). It is therefore likely that adrenarche is a complex process involving a finely tuned interplay between several enzymatic activities in the adrenal gland.

### CLASSICAL 3 $\beta$ -HYDROXYSTEROID DEHYDROGENASE DEFICIENCY

Classical 3 $\beta$ -HSD deficiency is a rare form of congenital adrenal hyperplasia (CAH) accounting for about 1% to 10% of cases of CAH (Bois *et al.*, 1985; Thilen and Larsson, 1990). The salt-losing forms of CAH are a group of life-threatening diseases that require prompt recognition and treatment. Indeed, autosomal recessive mutations in the CYP21, CYP17, CYP11B1 and HSD3B2 genes that encode steroidogenic enzymes, in addition to mutations in the gene encoding the intracellular cholesterol transport protein StAR, can cause CAH, each resulting in different biochemical consequences and clinical features (Forest 1995; Donohoue, 1995; Wilson and New, 1998; Grumbach and Conte, 1999). These five biochemical defects impair cortisol secretion, which results in compensatory hypersecretion of ACTH and consequent hyperplasia of the adrenal cortex. However, only deficiencies in 21-hydroxylase (CYP21) and 11 $\beta$ -hydroxylase (CYP11B1) predominantly result in virilizing disorders. Indeed, in patients with the classical form of these two defects, the most noticeable abnormality in the sexual phenotype is the masculinization of the female fetus due to oversynthesis of adrenal DHEA and DHEA-S, whereas affected males do not have ambiguous genitalia. In contrast to these two most frequent causes of CAH, that are exclusive adrenal defects; 3 $\beta$ -HSD, CYP17 and StAR deficiencies impair steroidogenesis in both the adrenals and the gonads, resulting in the diminished formation of sex steroids in addition to cortisol and aldosterone.

#### Clinical features

There are two distinct classes of 3 $\beta$ -HSD deficiency, namely the classical and the non-classical forms. Since the first reports by Bongiovanni (1961, 1962) many patients of both sexes have been described that display heterogeneity upon clinical presentation (Table 8.1). Classical 3 $\beta$ -HSD deficiency results from mutations in the HSD3B2 gene, while the HSD3B1 gene in these patients is normal (Rheaume *et al.*, 1992; Simard *et al.*, 1993b, 1995; Russell *et al.*, 1994; Morel *et al.*, 1997; Pang, 1998; Wilson and New, 1998; Quigley, 1998; Moisan *et al.*, 1999; and references therein). The classical form of 3 $\beta$ -HSD deficiency can be divided, depending upon the severity of the salt-wasting associated with the disorder, into the salt-

wasting or non-salt wasting forms. There is no correlation between the impairment in male sexual differentiation and salt wasting.

#### *At age of diagnosis*

Overall, male individuals suffering from classical 3 $\beta$ -HSD deficiency show either perineal hypospadias or perineoscrotal hypospadias, as shown in Table 8.1. On the other hand, complete or partial inhibition of 3 $\beta$ -HSD activity in the adrenals and ovaries is not accompanied by a noticeable alteration in differentiation of the external genitalia of female patients, as indicated by the absence of ambiguity of the external genitalia. The reason for this striking difference in male and female phenotype is that deficiency of 3 $\beta$ -HSD in the fetal testis results in a lowering of T levels, which are essential for the normal development of external genitalia as indicated earlier (Rheaume *et al.*, 1992; Simard *et al.*, 1993b, 1995; Russell *et al.*, 1994; Morel *et al.*, 1997; Pang, 1998; Wilson and New, 1998; Quigley, 1998). However, males affected with pseudohermaphroditism and complete or partial 3 $\beta$ -HSD deficiency have intact Wolffian duct structures, including the vas deferens. This is also the case in 17 $\beta$ -HSD type 3 deficiency as well as 5 $\alpha$ -reductase type 2 deficiency, which is consistent with the hypothesis that a principal effect of 3 $\beta$ -HSD deficiency is to reduce the formation of DHT below the level required for the normal development of external genitalia (Russell *et al.*, 1994). The salt-losing form of classical 3 $\beta$ -HSD deficiency is usually diagnosed during the first few months of life due to insufficient biosynthesis of aldosterone and the consequent salt loss that may be fatal if not diagnosed and treated early (Bongiovanni, 1961; Zachmann *et al.*, 1970; Janne *et al.*, 1970; Kenny *et al.*, 1971; Parks *et al.*, 1971; de Peretti *et al.*, 1980; Heinrich *et al.*, 1993; Quigley, 1998). In contrast, the non salt-losing form of 3 $\beta$ -HSD deficiency may be diagnosed either at a young age in the presence of indicating factors, such as a family history of death during early infancy (Gendrel *et al.*, 1979), perineal hypospadias in male newborns (Rosenfield *et al.*, 1980; Nahoul *et al.*, 1989), failure to gain weight (de Peretti *et al.*, 1980), or the diagnosis may be made at a later date (Pang *et al.*, 1983; Cara *et al.*, 1985; Mendonca *et al.*, 1987; Heinrich *et al.*, 1993). Due to the fact that sexual differentiation is normal in female newborns affected by non salt-losing 3 $\beta$ -HSD deficiency, the correct diagnosis is delayed until adrenarche (Mendonca *et al.*, 1987; Alos *et al.*, 2000) or puberty (Cara *et al.*, 1985) (Table 8.1). It has recently been postulated that the under-representation of 46XX patients for this autosomal recessive disease suggests that 46XX individuals, because they do not in general occur as intersex newborns, are not diagnosed correctly and/or may die of an adrenal crisis prior to diagnosis more often than 46XY individuals (Alos *et al.*, 2000). In a 1984 survey, Lafont noted that, out of 20 patients with the severe salt-wasting form of 3 $\beta$ -HSD deficiency, only 6 were genetic females. Interestingly, the same sex ratio is found in the cases that have now been confirmed by mutation analysis, with only 9 female individuals out of a total number of 29 patients, as shown in Table 8.1. This is a mirror image of what occurs in the more common 21-hydroxylase deficiency, in which there is an under-representation of clinically diagnosed 46XY individuals (Donohoue, 1995). It is also of interest to note that among the 9 females suffering from the non salt-losing form of the disease, 6 were Brazilian (Table 8.1).

**Table 8.1** Genotype-phenotype relationships of patients with classical 3 $\beta$ -HSD deficiency bearing a missense, nonsense, frameshift or an in-frame deletion mutation in the HSD3B2 gene. The ethnic origin, the phenotypic characteristics and clinical features of the patients are indicated as well as a summary of the functional consequence of mutations, or sequence variants in the HSD3B2 gene. The mutations previously designated 186/insC/187, 266AA, 273AAA, and 318AC have been changed to 558insC, 818delAA, 797delA and 953delC, respectively, to follow the current international nomenclature where the first nucleotide used to assign the name of the mutant is the A of the ATG codon. The apparent activity of mutant enzymes in intact cells was calculated after a 1-hour incubation. The values indicated are the mean  $\pm$  SEM of two independent experiments performed in triplicate.

Family	Origin	Patient	Karyotype	Age at diagnosis	Phenotype	Case report/ Mutation report	First functional characterization report	Mutant alleles	Apparent activity in intact cells	Evidence of protein instability	Conservative residue in 3 $\beta$ -HSD family
1	French- Canadian	1	46XX	3 weeks	Normal genitalia, Breast development and menses at puberty	(Alos <i>et al.</i> , 2000)	(Alos <i>et al.</i> , 2000)	A10E/ A10E	No detectable activity	Yes	Gly in bovine and pig
2	French- Canadian	2	46XY	13 days	Ambiguous genitalia Palpable testes in bifid scrotum	(Alos <i>et al.</i> , 2000)	(Alos <i>et al.</i> , 2000)	A10E/ A10E	No detectable activity	Yes	Gly in bovine and pig
3	Algerian	3	46XY	Birth	Perineoscrotal hypospadias, Bifid scrotum	(Gendrel <i>et al.</i> , 1979; Rheume <i>et al.</i> , 1995)	(Rheume <i>et al.</i> , 1995)	G15D/ G15D	No detectable activity	Yes	All vertebrate species
4	Spanish/ Portuguese	4	46XY	Birth	Perineal hypospadias, bifid scrotum	(de Peretti, 1980; LaFont, 1984; Sanchez <i>et al.</i> , 1994c)	(Sanchez <i>et al.</i> , 1994c)	L108W	No detectable activity	Yes	All vertebrate species
5	American	5	46XY	1 week	Perineal hypospadias, bifid scrotum	(Cara <i>et al.</i> , 1985; Simard <i>et al.</i> , 1993)	(Simard <i>et al.</i> , 1993b)	E142K	No detectable activity	No	All mammalian species except 3-KSRs and Scr in chicken All vertebrate species
								W171X	No predicted activity	N/A	N/A

6	Swiss	6	46XX	14.7 years	Lack of spontaneous breast development	(Zachmann <i>et al.</i> , 1970; Zachmann, 1979; Rheume <i>et al.</i> , 1992)	(Rheume <i>et al.</i> , 1992)	W171X/ W171X	No predicted activity	N/A	N/A
7	Swiss	7	46XX	?	Lack of spontaneous breast development	(Zachmann, 1988; Rheume <i>et al.</i> , 1992)	(Rheume <i>et al.</i> , 1992)	W171X/ W171X	No predicted activity	N/A	N/A
8	American	8	46XY	34 years	Hypospadias (1 month), Gynecomastia (13 years)	(Parks <i>et al.</i> , 1971; Rheume <i>et al.</i> , 1992)	(Rheume <i>et al.</i> , 1992)	W171X	No predicted activity	N/A	N/A
9	Chilian	9	46XX	Birth	Hyperpigmentation, Normal genitalia	(Kasumata <i>et al.</i> , 1995)		558insC	No predicted activity	N/A	N/A
10	Japanese	10	46XY	3 months	Hyperpigmentation, Severe hypospadias, Bifid scrotum	(Marui <i>et al.</i> , 1998a)	(Moisan <i>et al.</i> , 1999)	E135X/ E135X L205P/ L205P	No predicted activity No detectable activity	N/A No	N/A Val in hamster I or Ile in hamster II and trout
11	Algerian	11	46XX	11 months	Hyperpigmentation, mild clitoromegaly		(Moisan <i>et al.</i> , 1999)	P222Q/ P222Q	No detectable activity	No	Ser in hamster III
		12	46XY	Birth	Perineal hypospadias with micropenis						
		13	46XX	1 month	Palpable testes in scrotum Mild clitoromegaly First diagnosis 21-hydroxylase deficiency						

Family Origin	Patient	Karyotype	Age at diagnosis	Phenotype	Case report/ Mutation report	First functional characterization report	Mutant alleles	Apparent activity in intact cells	Evidence of protein instability	Conservative residue in 3 $\beta$ -HSD family
12 Japanese	14	46XY	N/A	Pigmentation, Hypospadias, Bifid scrotum	(Tajima <i>et al.</i> , 1995)		R249X/ R249X	No predicted activity	N/A	N/A
13 Japanese	15	46XY	N/A	Pigmentation, Mild clitoromegaly	(Tajima <i>et al.</i> , 1995)		R249X/ R249X	No predicted activity	N/A	N/A
14 Japanese	16	46XY	Birth	Hypospadias with micropenis	(Yoshimoto <i>et al.</i> , 1988; Yoshimoto <i>et al.</i> , 1997)		R249X/ R249X	No predicted activity	N/A	N/A
15 Dutch	17	46XY	Birth	Urethral diverticula and hypospadias	(Wolthers <i>et al.</i> , 1987; Van seters <i>et al.</i> , 1989; Simard <i>et al.</i> , 1993b)	(Simard <i>et al.</i> , 1993b)	Y253N 558insC	No detectable activity	Yes	All vertebrate species N/A
16 Taiwanese	18	46XY	Birth	Hypospadias Microphallus	(Zhang <i>et al.</i> , 2000)	(Moisan <i>et al.</i> , 1999; Zhang <i>et al.</i> , 2000)	T259M/ T259M	Very weak activity	Yes	All vertebrate species
17 French	19	46XY	Birth	Perineal hypospadias, Palpable testes in bifid scrotum	(Moisan <i>et al.</i> , 1999)	(Moisan <i>et al.</i> , 1999)	T259M	Very weak activity	Yes	All vertebrate species
	20	46XX	Birth	Normal external genitalia			867delG	No predicted activity	N/A	N/A
18 Japanese	21	46XY	N/A	Pigmentation, Hypospadias, Bifid scrotum	(Tajima <i>et al.</i> , 1995)	(Moisan <i>et al.</i> , 1999)	T259R/ T259R	No detectable activity	Yes	All vertebrate species
	22	46XX	2 weeks	Normal genitalia with severe pigmentation						

19	Japanese	23	46XY		Pigmentation, Perineal hypospadias	(Tajima <i>et al.</i> , 1995)	Y308X	No predicted activity	N/A	N/A
20	Sri-Lankan	24	46XY	Birth	Perineal hypospadias with micropenis, Palpable testes in scrotum	(Moisan <i>et al.</i> , 1999)	687del27/ 687del27	No detectable activity	Yes	N/A
21	Sri-Lankan	25	46XY	Birth	Perineal hypospadias	(Moisan <i>et al.</i> , 1999)	687del27/ 687del27	No detectable activity	Yes	N/A
22	Afghan/ Pakistani	26	46XY	2 weeks	Perineal hypospadias	(Simard <i>et al.</i> , 1994)	818delAA/ 818delAA	No predicted activity	N/A	N/A
23	Afghan/ Pakistani	27	46XY	Birth	Ambiguous genitalia Hypospadias, Bifid scrotum	(Simard <i>et al.</i> , 1994)	818delAA/ 818delAA	No predicted activity	N/A	N/A
24	Afghan/ Pakistani	28	46XY	Birth	Hypospadias Bifid scrotum	(Simard <i>et al.</i> , 1994)	818delAA/ 818delAA	No predicted activity	N/A	N/A
25	Pakistani	29	46XX	Birth	Pigmentation, Mildly enlarged clitoris	(Zhang <i>et al.</i> , 1996)	818delAA/ 953delC	No predicted activity Not determined	N/A	N/A



Family	Origin	Patient	Karyotype	Age at diagnosis	Phenotype	Case report/ Mutation report	First functional characterization report	Mutant alleles	Apparent activity in intact cells	Evidence of protein instability	Conservative residue in $\beta$ -HSD family
<i>Non-salt-wasting form (elevated renin activity)</i>											
26	French	30	46XY	20 months	Perineal hypospadias	(de Peretti <i>et al.</i> , 1980; Mebarki <i>et al.</i> , 1995)	(Mebarki <i>et al.</i> , 1995)	N100S/ N100S	2.8 $\pm$ 0.07%	No	All vertebrate species
27	English	31	46XY	9 years	Perineal hypospadias with normal testes, initial diagnosis of 21-hydroxylase deficiency (elevated renin)	(Fisher <i>et al.</i> , 1988; McCartin <i>et al.</i> , 2000)	(Mebarki <i>et al.</i> , 1995)	N100S	1.3%	No	All vertebrate species
								797delA	No predicted activity	N/A	N/A
		32	46XY	11 years	Perineal hypospadias, Micropenis with normal testes (normal renin)						
28	French	33	46XY	Birth	Perineal hypospadias, Palpable testes in scrotum	(Gendrel <i>et al.</i> , 1979; Moisan <i>et al.</i> , 1999)	(Moisan <i>et al.</i> , 1999)	P155L/	No detectable activity	No	Ala in hamster III and chicken; Ser in trout Ala, Ser or Cys
		34	46XY	Birth	Perineal hypospadias, Palpable testes in scrotum			G294V	20.5 $\pm$ 4.6%	No	

29	Brazilian	35	46XX	7 years	Normal genitalia Premature pubarche	(Marui <i>et al.</i> , 1998b)	(Moisan <i>et al.</i> , 1999)	P222H/ L6F	No detectable activity	No	Ser in hamster III
		36	46XX	6.7 years	Normal genitalia Premature pubarche		(Rheume <i>et al.</i> , 1994)	G129R	11.7 $\pm$ 0.1%	No	All vertebrate species
<i>Non-salt-wasting form (normal renin activity)</i>											
30	Pakistani	37	46XY	Birth	Hypospadias Hyper- pigmented and bifid scrotum	(Zhang <i>et al.</i> , 2000)	(Zhang <i>et al.</i> , 2000)	L6F/ L6F	54.75 $\pm$ 8%	No	Val in trout
31	Egyptian	38	46XY	6 months	Perineoscrotal hypospadias	(Moisan <i>et al.</i> , 1999)	(Moisan <i>et al.</i> , 1999)	A10V/ A10V	29.1 $\pm$ 0.6%	No	Gly in bovine and pig
		39	46XY	4 months	Perineoscrotal hypospadias						
32	Brazilian	40	46XX	31 years	Clinically normal	(Mendonca <i>et al.</i> , 1994; Mendonca <i>et al.</i> , 1987)	(Moisan <i>et al.</i> , 1999)	A82T/ A82T	7.6 $\pm$ 0.5%	No	All vertebrate species
		41	46XY	Birth	Ambiguous genitalia						
		42	46XY	Birth	Ambiguous genitalia						
33	Brazilian	43	46XX	5 years	Premature pubarche	(Mendonca <i>et al.</i> , 1994)	(Moisan <i>et al.</i> , 1999)	A82T/ A82T	7.6 $\pm$ 0.5%	No	All vertebrate species
34	American	44	46XX	18 years	Premature pubarche, Acne, Mild clitoromegaly	(Chang <i>et al.</i> , 1993b; Pang <i>et al.</i> , 1983; Rheume <i>et al.</i> , 1994)	(Rheume <i>et al.</i> , 1994)	G129R/ A82T	11.7 $\pm$ 0.1%	No	All vertebrate species
		45	46XY	16 years	Perineal hypospadias, bilateral cryptorchidism			6651 (G to A)	No predicted activity	N/A	N/A

Family Origin	Patient	Karyotype	Age at diagnosis	Phenotype	Case report/ Mutation report	First functional characterization report	Mutant alleles	Apparent activity in intact cells	Evidence of protein in $\beta$ -HSD family instability	Conservative residue	
35	Scottish	46	46XY	5 years	Perineal hypospadias	(Russell <i>et al.</i> , 1994)	(Moisan <i>et al.</i> , 1999)	L173R/ L173R	52.8 $\pm$ 0.6%	No	All vertebrate species
		47	46XX	2 years	Clinically normal						
36	French	48	46XY	Birth	Perineal hypospadias with micropenis	(Moisan <i>et al.</i> , 1999)	(Moisan <i>et al.</i> , 1999)	L286S/ 867delG	100% No predicted activity	No N/A	Ala, Ser N/A
37	Turkish	49	46XY	4 years	No palpable testes Scrotal hypospadias, bifid scrotum	(Heinrich <i>et al.</i> , 1993; Simard <i>et al.</i> , 1993b)	(Simard <i>et al.</i> , 1993)	A245P/ A245P	35.4 $\pm$ 0.2%	Yes	Ser, Val, Arg
38	American	50	46XX	Puberty	Primary amenorrhea, Slight clitoromegaly, Moderate hirsutism, Enlarged polycystic ovaries	(Rosenfield <i>et al.</i> , 1980; Barnes <i>et al.</i> , 1989; Barnes <i>et al.</i> , 1993; Sanchez <i>et al.</i> , 1994c)	(Sanchez <i>et al.</i> , 1993)	Y254D/ ?	No detectable activity N/A	No N/A	All vertebrate species N/A
39	Brazilian	51	46XX	31 years	Clitoromegaly, severe virilization	(Paula <i>et al.</i> , 1994)	(Moisan <i>et al.</i> , 1999)	T259M/ T259M	Very weak activity	Yes	All vertebrate species
40	Brazilian	52	46XX	7.8 years	Mild clitoromegaly	(Marui <i>et al.</i> , 1998b)	(Moisan <i>et al.</i> , 1999)	T259M/ T259M	Very weak activity	Yes	All vertebrate species

Sequence variants											
41	American	53	46XX	15.6 years	Hirsutism and/or oligomenorrhea	(Nayak <i>et al.</i> , 1998)	(Moisan <i>et al.</i> , 1999)	A167V	81.45 ± 0.03%	No	Gln, Glu,
42	American	54	46XX	7 years	Premature pubarche at 4 years, growth acceleration	(Chang <i>et al.</i> , 1993a)	(Moisan <i>et al.</i> , 1999)	S213G/	58.4 ± 0.6%	No	Gly, Lys
43	American	55	46XX	9 years	Premature pubic hair	(Nayak <i>et al.</i> , 1998)	(Moisan <i>et al.</i> , 1999)	K216E L236S	58.9 ± 0.2% 100%	No	Arg, Asn, Cys,
44	American	56	46XX	17 years	Hirsutism and/or oligomenorrhea	(Nayak <i>et al.</i> , 1998)	(Moisan <i>et al.</i> , 1999)	L236S	100%	No	Gly, His, Lys, Val
										No	Arg, Thr
										No	Ala, Ser
										No	Ala, Ser

*At puberty*

As stated above, very few cases of severe salt-wasting 3 $\beta$ -HSD deficiency have been diagnosed in genetic females and only Zachmann and colleagues (Zachmann *et al.*, 1970, 1979) have reported a follow-up at pubertal age in a patient who showed only minimal breast development at 14.7 years of age. She was treated with gluco- and mineralocorticoids and showed no increase in plasma T, estradiol or estrone levels after 5 days of gonadotrophin injections, and required estrogen treatment in order to undergo complete feminization, including menses. Moreover, in the absence of sex steroid replacement, she had continuously elevated LH and FSH levels and never showed any signs of ovulation, whereas after stopping estrogen/ progesterone treatment in adulthood her menses ceased and she developed multiple ovarian cysts (M.Zachmann, personal communication; Alos *et al.*, 2000). In contrast, the patient recently reported by Van Vliet underwent progressive feminization starting between 8 and 9 years of age (Alos *et al.*, 2000), and she was the first female with severe salt-wasting 3 $\beta$ -HSD deficiency reported to have developed progressive breast development, regular menses and evidence of progesterone secretion. It is worth mentioning that both the patient described by Zachmann *et al.*, (1970, 1979) and the patient reported by Van Vliet were homozygous carriers for a deleterious mutation (Rheaume *et al.*, 1992; Alos *et al.*, 2000) that results in the complete loss of type II 3 $\beta$ -HSD activity. One possible explanation for the development of breasts and the endometrium in the patient reported by Van Vliet is that there is local conversion of inactive adrenal precursors into estrogens. On the other hand, it is possible that, as suggested for the testes (Yoshimoto *et al.*, 1997), pubertal levels of gonadotropins may induce sufficient 3 $\beta$ -HSD activity by increasing the normally low levels of type I 3 $\beta$ -HSD expressed in the ovary, thereby allowing significant ovarian production of estradiol. The rise in the patient's plasma progesterone levels favors the latter hypothesis and raises the possibility that this individual may be fertile (Alos *et al.*, 2000). Interestingly, a male proven to be affected with severe 3 $\beta$ -HSD deficiency has in fact fathered children (Parks *et al.*, 1971; Rheaume *et al.*, 1992). The recent findings further illustrate that genotype may not always predict the phenotype in patients with this disorder. In this regard, it is also relevant to discuss the difference in phenotypic expression of a missense mutation responsible for the non salt-losing form of classical 3 $\beta$ -HSD deficiency associated with pseudohermaphroditism in genetic males and premature pubarche or cryptic expression in genetic females originating from Brazil (patients 40 and 43, Table 8.1) (Mendonca *et al.*, 1987, 1994). Patient 40, a genetic female, was phenotypically normal and was 31 years old when the case was first reported, with pubarche at the normal age and regular menstrual cycles. There was no clitoral enlargement, no hirsutism, and pubic hair and breast development were both at Tanner stage V. Analysis of urinary steroids and serum steroid concentrations revealed 3 $\beta$ -HSD deficiency in this index case. In the other Brazilian family, 33, which was unrelated to family 32, a 5-year-old girl (46XX) who displayed pubic hair (Tanner stage III) and acne was found to be homozygous for the same mutation. Her bone age, height, weight and breast development were normal for her age, and in addition, she had normal external genitalia and steroid measurements confirmed 3 $\beta$ -HSD deficiency in this patient. It was therefore of interest to observe that both this latter female patient and index case 40 were homozygous for the A82T mutation and showed similar alterations in serum steroid levels indicative of

3 $\beta$ -HSD deficiency, although only the index case from family 33 had premature pubarche. Thus, the lack of clinical symptoms in index case 40 demonstrated that the A82T mutation may be phenotypically cryptic in certain patients, but can have clinical effects in others (Mendonca *et al.*, 1994; Russell *et al.*, 1994).

## Biological diagnosis

### *Classical 3 $\beta$ -HSD deficiency*

The basal plasma levels of  $\Delta^5$ -3 $\beta$ -hydroxy steroids such as PREG, 17OH-PREG and DHEA are elevated in affected individuals. An elevated ratio of  $\Delta^5/\Delta^4$ -steroids is considered to be the best biological parameter for the diagnosis of 3 $\beta$ -HSD deficiency (de Peretti and Forest, 1982; Cara *et al.*, 1985). Following extensive sequencing of DNA from subjects suspected to be suffering from 3 $\beta$ -HSD deficiency, due to an elevated ratio of  $\Delta^5/\Delta^4$  steroids, the best criteria for the correct diagnosis of this disorder now appears to be a plasma level of 17OH-PREG greater than 100 nmol/L following stimulation with ACTH. It is well recognized, however, that levels of 17OH-PROG and  $\Delta^4$ -DIONE plasma and other  $\Delta^4$ -steroids are frequently elevated in 3 $\beta$ -HSD-deficient patients (Bongiovanni, 1962; Janne *et al.*, 1970; Zachmann *et al.*, 1970; Pang *et al.*, 1983; Cara *et al.*, 1985; Mendonca *et al.*, 1987; Rheaume *et al.*, 1992; Simard *et al.*, 1993b, 1995; Russell *et al.*, 1994; Morel *et al.*, 1997; Pang, 1998; Wilson and New, 1998; Quigley, 1998; Labrie *et al.*, 1999). The T response to hCG is usually poor in infancy (de Peretti *et al.*, 1980) but may be substantial in pubertal boys (Rosenfield *et al.*, 1974). Such observations are consistent with a functional type I 3 $\beta$ -HSD that is expressed in peripheral tissues and is responsible for the extra-adrenal and extra-gonadal conversion of  $\Delta^5$ -hydroxysteroid precursors into their corresponding  $\Delta^4$ -3-ketosteroids (reviewed in Labrie, 1997, 1991; Labrie *et al.*, 1997, 1999). Indeed, the 3 $\beta$ -HSD activity and/or type I 3 $\beta$ -HSD expression have been reported in several normal and tumoral peripheral tissues in humans and primates including the skin, breast, endometrium, myometrium, prostate, epididymis, adipose tissue, liver and colon, as described above (Rheaume *et al.*, 1991; Dumont *et al.*, 1992; Martel *et al.*, 1994; Gingras and Simard, 1999; Gingras *et al.*, 1999; and references therein). It is also relevant to mention that although very low levels of type I 3 $\beta$ -HSD mRNA can be detected in normal gonads using the sensitive ribonuclease protection assay,  $\Delta^4$ -steroids can originate from gonadal 3 $\beta$ -HSD type I activity, which possesses a roughly 5 to 10-fold higher affinity for the substrate than the type II isoenzyme. This could be stimulated following an increase in LH secretion, resulting from low circulating androgen levels at puberty (Pang *et al.*, 1985; Rheaume *et al.*, 1991, 1992). Indeed, Yoshimoto and colleagues (Yoshimoto *et al.*, 1997) have recently shown that pubertal levels of gonadotropins may increase intra-testicular 3 $\beta$ -HSD activity in spite of the low levels of HSD3B1 transcripts. However, the peripheral type I 3 $\beta$ -HSD activity could explain why certain patients were initially mis-diagnosed to be suffering from 21-hydroxylase deficiency, in view of the elevated levels of 17OH-PROG and mild virilization seen in girls at birth (Morel *et al.*, 1997). Nevertheless, the significant accumulation of precursors, for example 17OH-PREG, may also interfere with the radioimmuno-assays used routinely in laboratories to measure levels of 17OH-PROG, although in practice this

elevated level of 17OH-PROG is considered beneficial. Indeed, an increased level of 17OH-PROG observed when screening neonates for 21-hydroxylase deficiency should help in the correct diagnosis of 3 $\beta$ -HSD deficiency. Therefore, measurement of the levels of 17OH-PREG should be performed when an elevated level of 17OH-PROG has been observed in a female neonate without ambiguity of external genitalia, or if the patient is a male pseudohermaphrodite. In agreement with this suggestion, during the French neonatal screening period from 1989–1999 two patients suffering from a salt-wasting form of 3 $\beta$ -HSD deficiency were detected (patient 20, Table 8.1, and a third patient originating from Sri-Lanka; Morel, unpublished results). Because the correct diagnosis of 3 $\beta$ -HSD deficiency at birth is of most importance, the basal levels of the steroids associated with, or without, the ACTH-stimulation test were sufficient. Nevertheless, plasma renin levels should always be considered in order to evaluate any appreciable salt-loss. Furthermore, an hCG test in a male individuals showed a decrease in T and  $\Delta^4$ -DIONE plasma levels, which is associated with an increase in DHEA levels.

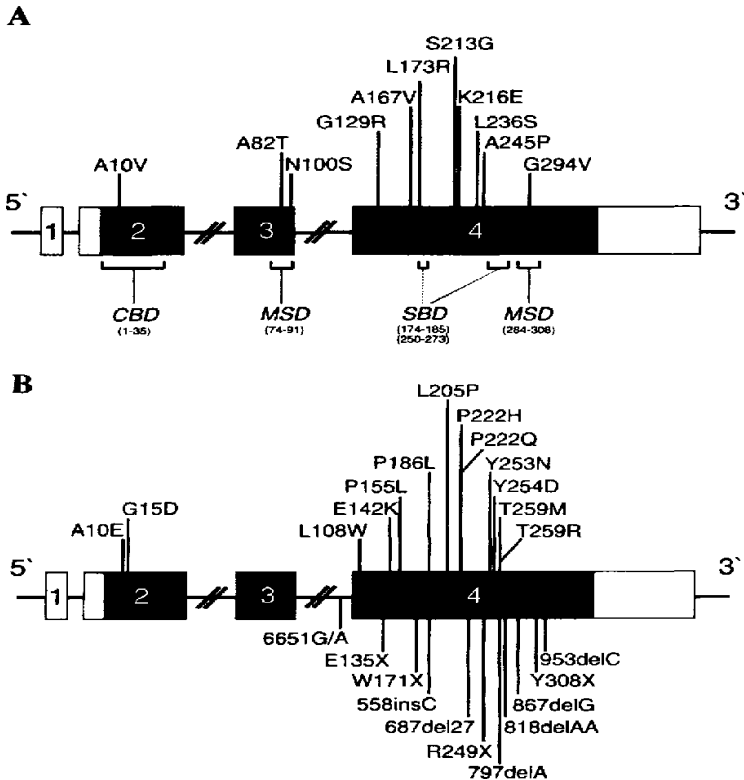
#### *Non-classical 3 $\beta$ -HSD deficiency*

In the presence of premature pubarche with high DHEA-S levels in girls, the diagnosis of non-classical 3 $\beta$ -HSD deficiency was often evoked. The non-classical form of 3 $\beta$ -HSD deficiency, also referred to as attenuated or late-onset deficiency, has been described in older females with hyperandrogenism beginning at adulthood and in children with premature pubarche (Pang *et al.*, 1985; Medina *et al.*, 1986; Eldar-Geva *et al.*, 1990; Schram *et al.*, 1992; Zerah *et al.*, 1994). No mutation was found in either the HSD3B1 and/or HSD3B2 genes in these patients (Zerah *et al.*, 1994; Chang *et al.*, 1995; Forest *et al.*, 1995; Sakkal-Alkaddour *et al.*, 1996), and upon re-examination certain patients no longer showed an elevated  $\Delta^5/\Delta^4$  ratio (Zerah *et al.*, 1994; Forest, 1995). Moreover, Morel's group was also unable to find any mutations in either the HSD3B1 and HSD3B2 genes in 20 girls having a peak level of 17OH-PREG between 30 to 90 nmol/L following ACTH stimulation (Mebarki, 1995; Morel *et al.*, 1997). Consequently, a post ACTH-stimulation value of 17OH-PREG was always required in order to establish the correct diagnosis of 3 $\beta$ -HSD deficiency. Further sequencing of the HSD3B2 gene in patients showing a post-ACTH-stimulation value of 17OH-PREG between 30 to 90 nmol/L will therefore be necessary to confirm that 100 nmol/L should be the cut-off value leading to the diagnosis of classical 3 $\beta$ -HSD deficiency, a suggestion also recently made by another group (Marui *et al.*, 2000). There is accumulating evidence proposed by Pang and colleagues (Pang *et al.*, 1985) suggesting that the hormonal criteria for the bona fide diagnosis of the non-classical variants of 3 $\beta$ -HSD deficiency should be increased or reappraised. This includes the post ACTH-stimulated 17OH-PREG and DHEA levels and the ratios of 17OH-PREG/ 17OH-PROG, 17OH-PREG/cortisol or DHEA/ $\Delta^4$ -DIONE which should be >2SD above the pubertal stage-matched normal mean values (Pang, 1997). Finally, it has been concluded that it is difficult, if not impossible, to provide any kind of accurate statement regarding the clinical features, pathophysiology or diagnosis of this particular disorder (Moran *et al.*, 1998).

## Molecular diagnosis

*No mutation in the HSD3B1 gene*

To identify the molecular lesions in the genes encoding type I and/or type II  $3\beta$ -HSD responsible for the salt-losing form of classical  $3\beta$ -HSD deficiency, the nucleotide sequence



**Figure 8.5** Schematic representation of all the mutations identified to date in the **HSD3B2** gene in individuals suffering from classical  $3\beta$ -HSD deficiency. Figure (A) represents the missense mutations that have been shown to retain a certain amount of  $3\beta$ -HSD activity. Also highlighted is the cofactor binding domain (CBD), the two putative substrate binding domains (SBD) and the two membrane spanning domains (MSD). Figure (B) represents the missense mutations that have been shown to abolish enzyme activity (upper panel), and the nonsense, frameshift and in-frame deletion mutations (lower panel).

of the whole coding region, exon-intron splicing boundaries as well as the 5'-flanking region, including the putative promoter and the 3'-non-coding region of each of the two genes, were determined by direct sequencing. The complete sequence of the PCR products obtained from the **HSD3B1** gene from six patients originating from five unrelated families was identical to that of the corresponding known regions in the normal gene (Rheume *et al.*, 1992; Simard *et al.*, 1993b; Sanchez *et al.*, 1994b), therefore providing no evidence to



suggest a genetic alteration affecting type I mRNA processing or a structural change in the type I 3 $\beta$ -HSD isoenzyme in these patients. In agreement with this finding, no mutation was detected in the HSD3B1 gene in three unrelated patients with the non salt-losing form of classical 3 $\beta$ -HSD deficiency (Simard *et al.*, 1993b; Rheume *et al.*, 1994; Mebarki *et al.*, 1995). As described above, the high production rate of progesterone by the placenta due to type I 3 $\beta$ -HSD activity is essential for the maintenance of human pregnancy. Consequently it was suggested that in a fetus homozygous for an HSD3B1 gene defect, the absence of placental enzyme activity would lead to interruption of pregnancy before the end of the first trimester (Rheume *et al.*, 1992; Simard *et al.*, 1993b, 1995).

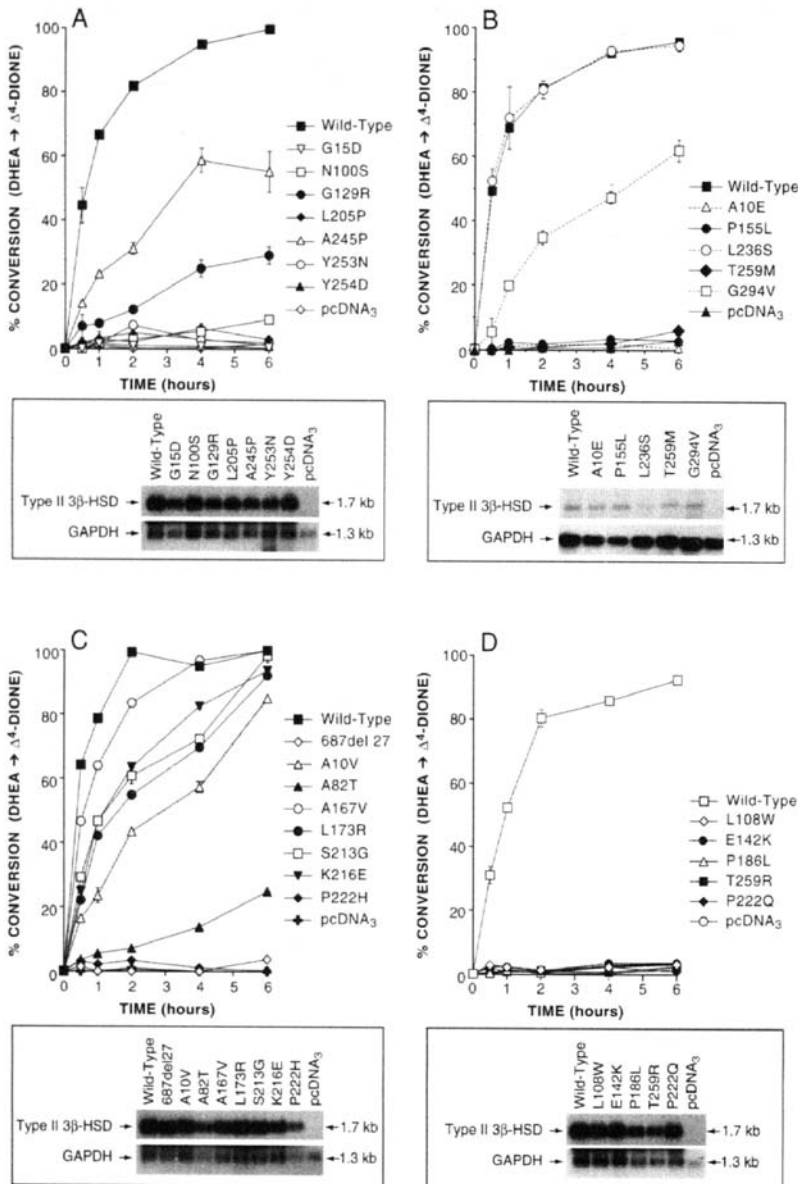
#### *Detection of mutations in the HSD3B2 gene*

To date a total of 34 mutations (including 5 frameshift, 4 nonsense, 1 in-frame deletion, 1 splicing and 23 missense mutations) have been identified in the HSD3B2 gene in 56 individuals from 44 families suffering from classical 3 $\beta$ -HSD deficiency as shown in Table 8.1 and Figure 8.5. The functional significance of all the missense mutations identified to date in the HSD3B2 gene has been characterized (Simard *et al.*, 1995; Moisan *et al.*, 1999; Zhang *et al.*, 2000). In almost every case, the characterization of HSD3B2 mutations has provided a molecular explanation for the heterogeneous clinical presentation of this disorder (Moisan *et al.*, 1999).

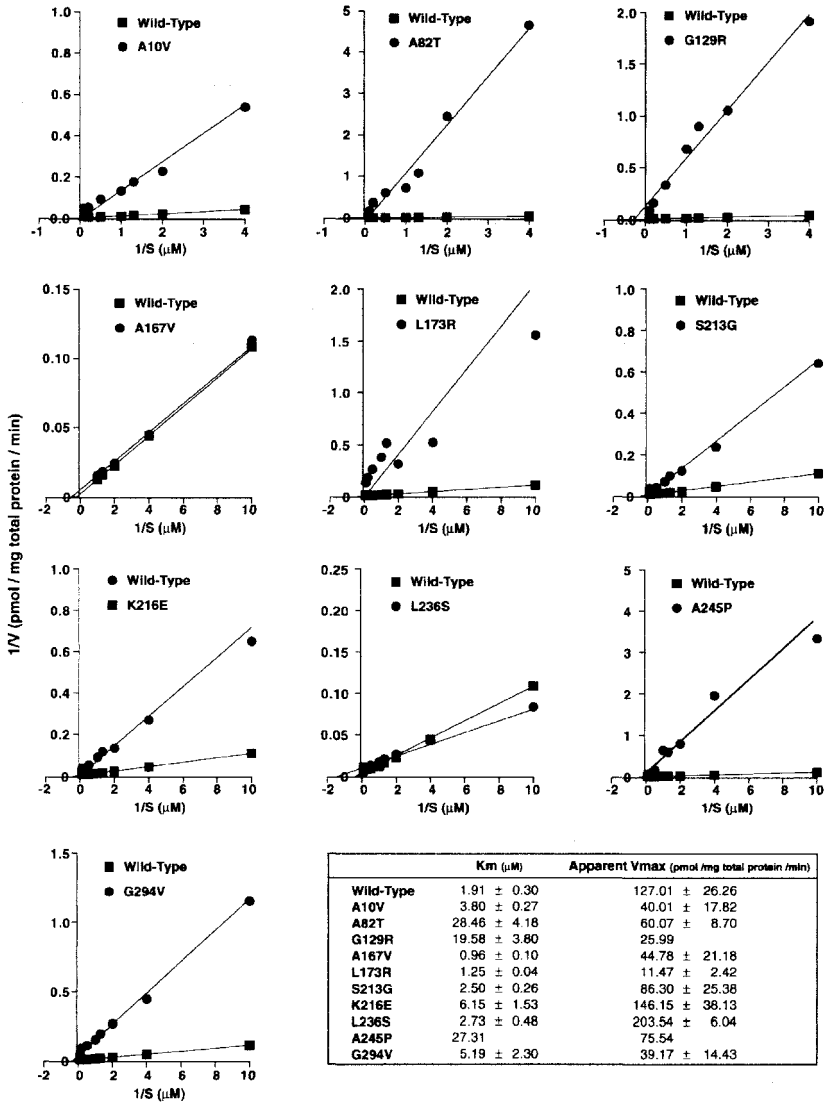
### **Functional characterization of type II 3 $\beta$ -HSD mutant proteins**

#### *Advantages of using intact cells to assess enzymatic characteristics*

The data recently presented by Moisan and colleagues supports the notion that it is more appropriate to assess the enzymatic activity of transiently expressed mutant proteins using intact 293 cells rather than homogenates from cells, due to the fact that addition of exogenous co-factor can drive a reaction that may not occur *in vivo* (Figures 8.6 and 8.7) (Moisan *et al.*, 1999). For example, as shown in Table 8.2 enzyme activity can be detected for mutants L108W and P186L when assessed in cell homogenates, using PREG as the substrate, while no activity can be detected in intact transfected cells using DHEA as the substrate. This conclusion is also consistent with a previous report showing that the homozygous mutation G15D, identified in a patient suffering from a severe salt-wasting form of the disease, completely abolished the activity of the transiently expressed mutant protein in intact cells, whereas significant residual enzyme activity could be observed when using cell homogenates (Rheume *et al.*, 1995). The use of an excessive amount of exogenous co-factor in studies using cell homogenates was responsible, at least in part, for this apparent discrepancy because this mutation, which is located in the NAD-binding domain, markedly decreases the affinity for the co-factor. Mutations N100S, L108W and P186L also severely affect the affinity for the co-factor (Sanchez *et al.*, 1994b; Mebarki *et al.*, 1995), as shown in Table 8.2. On the other hand, it is worth noting that the kinetic properties of the N100S mutant protein, found in a non salt-losing patient (Sanchez *et al.*,



**Figure 8.6** Comparison of the time course of enzymatic conversion of [ $^{14}$ C]-DHEA into [ $^{14}$ C]- $\Delta^4$ -DIONE in intact 293 cells in culture transfected with the indicated expression vectors. The results are presented as the mean  $\pm$  SEM ( $n=3$ ). When the SEM overlaps with the symbol used, only the symbol is illustrated. Inset: Northern blot analysis demonstrating that following transient expression with the indicated expression vector constructs all transcripts were expressed at equal levels in transfected 293 cells. The cells were transfected with the pcDNA<sub>3</sub> vector alone to show no endogenous expression of type II  $3\beta$ -HSD mRNA. Hybridization to human GAPDH is also shown to act as a control (Moisan *et al.*, 1999).



**Figure 8.7** Comparison of the kinetic properties of mutant recombinant proteins with type II 3β-HSD activity following transient expression in intact 293 cells. The transiently transfected 293 cells were incubated with 10nM up to 50μM DHEA including 10nM [4-<sup>14</sup>C(N)]-DHEA and various concentrations of unlabeled DHEA for 15 min (wild-type, A167V, L236S), 30 min (L173R, K216E, S213G), 1 hour (A10V, A245P, G294V), 2 hours (G129R) or 3 hours (A82T) to establish the apparent  $K_m$  and  $V_{max}$  for each mutant protein compared to the wild-type. The data are displayed as Lineweaver-Burk plots. Under the conditions employed, first order kinetics were always maintained. The results are expressed as the mean ± SEM of at least three separate transfection experiments performed in triplicate, with the exception of data obtained with A245P, which was performed on a single occasion. The relative expression levels for all constructs were confirmed by Northern blot analysis as described in the legend of Figure 8.6, thus the  $V_{max}$  values were calculated using the amount of total cellular protein in each corresponding petri-dish following transfection experiments (Moisan *et al.*, 1999).

**Table 8.2** Kinetic parameters of wild-type II 3 $\beta$ -HSD and G15D, G15A, N100S, L108W and P186L mutant proteins assessed in cell homogenates using PREG as the substrate and NAD<sup>+</sup> as the co-factor (Sanchez *et al.*, 1994b; Mebarki *et al.*, 1995; Rheaume *et al.*, 1995).

<i>Protein</i>	<i>K<sub>m</sub></i> <i>PREG</i>	<i>Relative</i> <i>V<sub>max</sub></i> <i>PREG</i>	<i>Relative</i> <i>V<sub>max</sub></i> <i>PREG</i> <i>K<sub>m</sub>PREG</i>	<i>K<sub>m</sub></i> <i>NAD</i>	<i>Relative</i> <i>V<sub>max</sub></i> <i>NAD</i>	<i>Relative</i> <i>V<sub>max</sub></i> <i>NAD</i> <i>K<sub>m</sub>NAD</i>
Type II 3 $\beta$ -HSD	0.72	100	139	22	100	4.54
G15D	3.2	120	37.5	113	121	1.07
G15A	3.4	134	39.4	148	116	0.78
Type II 3 $\beta$ -HSD	1.7	100	58.8	24	100	4.2
L108W	12.0	16.1	1.3	678	17.4	0.025
P186L	18.0	20.7	1.2	920	17.4	0.019
Type II 3 $\beta$ -HSD	3.5	100	28.6	20	100	5
N100S	25	19.3	0.8	650	30	0.05
Type II 3 $\beta$ -HSD	2.6	100	38.5	–	–	–
A245P	4.6	20.3	4.4	–	–	–
Type II 3 $\beta$ -HSD	1	100	100	–	–	–
G129R	10	0.2	2.0	–	–	–

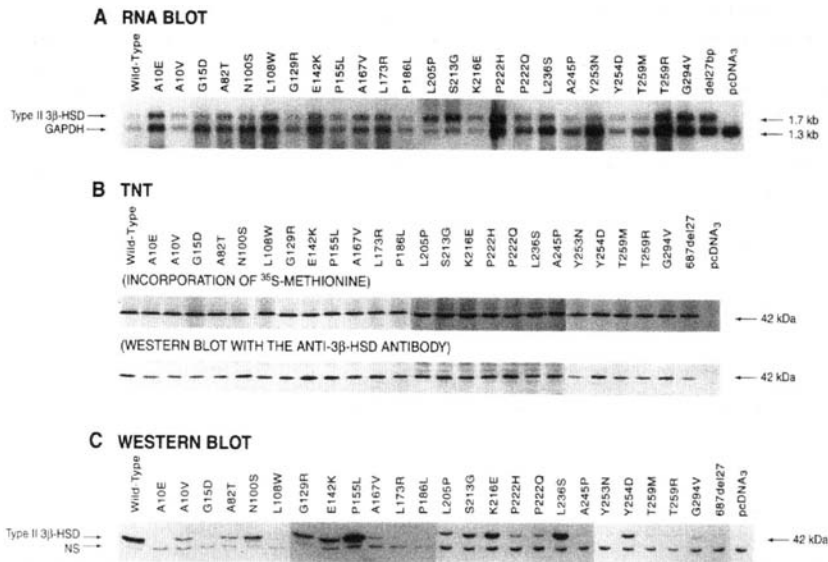
**Note**

Relative  $V_{\max}$  values were calculated assuming the  $V_{\max}$  for the human type II enzyme equal to 100.

1994c), are quite similar to those of the L108W and P186L mutant proteins, which were detected in an individual with a severe salt-losing form of classical 3 $\beta$ -HSD deficiency (Rheaume *et al.*, 1994), when the kinetic properties were measured using cell homogenates (Table 8.2). The functional characterization of these mutant proteins in cell homogenates cannot provide an explanation for the heterogeneity responsible for the severe salt-losing form (L108W:P186L) down to the clinically inapparent form of salt loss (N100S) of classical 3 $\beta$ -HSD deficiency. On the other hand, as illustrated in Figure 8.6, a significant level of enzyme activity was detectable in intact cells expressing the recombinant N100S mutant enzyme but not in those expressing the L108W and P186L mutant proteins. The advantage of using intact cells in order to assess enzymatic characteristics was also indicated by several previous studies describing the properties of numerous hydroxysteroid dehydrogenases. For example, the type 1 isozyme of 11 $\beta$ -hydroxysteroid dehydrogenase was always thought to act as both a dehydrogenase and an oxo-reductase, and it was not until assessment of enzyme activity in intact cells (Ricketts *et al.*, 1998; and references therein) that it became apparent that this isozyme behaves as a reductase *in vivo*, but *in vitro* can also act as a dehydrogenase due to the addition of exogenous co-factor (Ricketts *et al.*, 1998). A similar conclusion has been proposed concerning members of the 17 $\beta$ -HSD family (Peltoketo *et al.*, 1999). Furthermore, it was recently shown that the activity of the type 5 isozyme of 17 $\beta$ -HSD was unstable when assessed in homogenates of transfected cells compared to intact cells (Dufort *et al.*, 1999). However, we have previously observed in cells expressing the mutant protein A245P that no activity was obtained in cell homogenates in the absence of glycerol, while significant activity could be measured in intact cells (Simard *et al.*, 1993b).

*Apparent instability of mutant proteins associated with the salt-wasting form of  
3 $\beta$ -HSD deficiency*

In the past few years it has frequently been observed that it was difficult, if not impossible, to detect certain transiently expressed mutant recombinant proteins by Western blot analysis. Our recent study was designed to gain further biochemical data concerning this phenomena (Moisan *et al.*, 1999). In brief, all transcripts were expressed in transfected 293 cells following transient expression with the indicated expression vector constructs, as revealed by Northern blot analysis in both Figures 8.6 and 8.8A. In parallel, an *in vitro* transcription/translation (TNT<sup>®</sup>) rabbit reticulocyte lysate assay using the mutant cDNA constructs was performed to demonstrate that each pCDNA construct is adequately translated into a [<sup>35</sup>S]-labelled-42 kDa protein, indicative of the normal expression levels of mutant recombinant type II 3 $\beta$ -HSD proteins (Figure 8.8B). Western blot analysis of the corresponding samples from the TNT<sup>®</sup> assay hybridized with an anti-human type I 3 $\beta$ -HSD antibody indicated that all mutant proteins are recognized by this polyclonal antibody. The data illustrated in Figure 8.8B thus supports the notion that there is no detectable change on the polyclonal antibody binding site as a consequence of these mutations. Thereafter, Western blot analysis was performed using the homogenates purified from the same cells transfected with the indicated expression vector constructs, which have been used for RNA blot analysis illustrated in Figure 8.8A. A 42kDa band corresponding to the type II 3 $\beta$ -HSD protein was detectable in several but not all homogenate preparations from 293 transfected cells expressing the indicated wild type or mutant recombinant proteins (Figure 8.8C). The non specific band observed may also be used as an internal control for loading. Such a difference in immunoblot signal levels was observed in several independent experiments. Although the exact molecular and cellular explanation for the apparent instability of various mutant recombinant proteins in intact transfected 293 cells remains to be elucidated, our results illustrate that it might be difficult, if not impossible, to rigorously measure the levels of expression of some of these mutant proteins in order to obtain an accurate estimate of their  $V_{\max}$  value. We are thus suggesting that the various degrees of protein instability may explain, at least in part, not only the observed decrease in the  $V_{\max}$  values for several mutant proteins and more specifically for those with the L173R or G294V substitution (Figure 8.7), but also the absence of activity observed in 293 cells transiently expressing mutant recombinant proteins A10E, G15D, L108W, P186L, A245P, Y253N, T259M, T259R. For example, although the overall efficiency of the N100S protein found in patient 30, a homozygous non salt-loser (Table 8.2) (Mebarki *et al.*, 1995), and in two heterozygous carriers, patients 31 and 32, is very similar ( $\sim 0.1\%$ ) to that of L108W and P186L found in patient 4, a compound heterozygous salt-loser (Table 8.2) (Sanchez *et al.*, 1994b), the study by Moisan and colleagues (Moisan *et al.*, 1999) illustrates the inherent limitations of such experimental approaches. These should be used in conjunction to further evaluate the impact of a mutation on *in vivo* enzymatic activity. Indeed, the data suggesting the instability of mutants L108W and P186L, but not of mutant N100S, may well explain, at least in part, the observed difference in the levels of activity using intact cell assays, in accordance with the phenotypic differences observed in these patients. In fact, the present results suggest that a single amino acid substitution can alter the phenotypic expression of a protein by altering its stability. In agreement with this finding, it has previously been shown that a single amino



**Figure 8.8 Comparison of the levels of expression and stability of twenty-five mutant recombinant type II 3 $\beta$ -HSD proteins.** Panel A: Northern blot analysis demonstrating that following transient expression with the indicated expression vector construct all transcripts were expressed in transfected 293 cells. The cells were transfected with the pCDNA<sub>3</sub> vector alone to show no endogenous expression of type II 3 $\beta$ -HSD mRNA. Hybridization to GAPDH is also shown as a control of transfection efficiency. Panel B: Representation of an *in vitro* transcription/translation (TNT<sup>®</sup>) rabbit reticulocyte lysate assay using the mutant cDNA construct showing that each pCDNA<sub>3</sub> constructs is adequately translated into a [<sup>35</sup>S]-labelled-42kDa protein, indicative of the normal expression levels of mutant recombinant type II 3 $\beta$ -HSD proteins. Translation was assessed by separation on a 12% SDS-PAGE gel. To determine whether all mutant proteins are recognized by the polyclonal antibody, western blot analysis of the corresponding samples from the TNT<sup>®</sup> assay, probed with an anti-human type I 3 $\beta$ -HSD polyclonal antibody at 1:2000 dilution was performed as described (Moisan *et al.*, 1999). Panel C: Western blot analysis of the homogenates purified from the same corresponding transiently transfected 293 cells with the indicated expression vectors, which have been used for RNA blot analysis illustrated in the Panel A. A 42kDa band corresponding to the type II 3 $\beta$ -HSD protein was detectable in several but not all homogenate preparations from 293 transfected cells expressing the indicated wild type or mutant recombinant proteins, while no 42kDa protein is detected in cells transfected with the mock pCDNA<sub>3</sub> vector alone. The non specific band observed may also be used as an internal control for loading (Moisan *et al.*, 1999).

acid mutation in P4502C13, namely Ser<sup>180</sup> to Cys, located in a highly conserved region in the P4502C subfamily, determines a polymorphism by altering protein stability (Faletto *et al.*, 1992).

### Genotype-phenotype relationships

The current knowledge on the molecular basis of 3 $\beta$ -HSD deficiency is in agreement with the prediction that no functional type II 3 $\beta$ -HSD isoenzyme is expressed in the adrenals and gonads of the patients suffering from a severe salt-wasting form of CAH due to classical 3 $\beta$ -HSD deficiency (Table 8.1, Figure 8.6). Indeed, it can be predicted that in patients suffering from a severe salt-wasting form of 3 $\beta$ -HSD deficiency, who bear a homozygous E135X (Marui *et al.*, 1998a), W171X (Rheume *et al.*, 1992); R259X (Tajima *et al.*, 1995; Yoshimoto *et al.*, 1997); Y308X (Tajima *et al.*, 1995); 818delAA (Simard *et al.*, 1994)) or a compound heterozygous mutation W171X/558insC (Rheume *et al.*, 1992); 818delAA/953delC (Zhang *et al.*, 1996), these nonsense and frameshift mutations lead to truncated proteins. Consequently, the resultant mutant enzyme is expected to retain no activity. Furthermore, the missense mutations associated with the severe salt-wasting form of 3 $\beta$ -HSD deficiency (Figures 8.5 and 8.6) result in mutant 3 $\beta$ -HSD proteins that retain no detectable enzyme activity, e.g., A10E, G15D, L108W, E142K, P186L, L205P, P222Q, Y253N and T259R, as determined by incubation of intact 293 cells transfected with mutant constructs corresponding to each of the missense mutations identified to date (Table 8.1). This abolishment of enzyme activity is consistent with the phenotype of severe salt-wasting observed in patients with these particular mutations. Taken together, all of these results are in perfect agreement with the severity of this form of CAH. In addition, the current data demonstrate that the non salt-losing form of classical 3 $\beta$ -HSD deficiency also results from missense mutation (s) in the HSD3B2 gene, which causes an incomplete loss in enzyme activity, thus leaving sufficient activity to prevent salt-wasting (Simard *et al.*, 1993b, 1995; Sutcliffe *et al.*, 1996; Pang, 1998; Moisan *et al.*, 1999). Indeed, the hormonal profile of two of the individuals bearing the N100S mutation (patients 30 and 31) suggests that salt loss was compensated for by a limited capacity of aldosterone biosynthesis at the price of high renin synthesis (Table 8.1) (Mebarki *et al.*, 1995; McCartin *et al.*, 2000). In general, the present functional and biochemical data (Figures 8.6, 8.7 and 8.8) are in close agreement with the severity of the disease in patients suffering from the non salt-wasting form of 3 $\beta$ -HSD deficiency. In this respect, it can be estimated that the 3 $\beta$ -HSD activity catalyzed by the mutant type II proteins in the compound heterozygotes P155L/ G294V, P222H/G129R and G129R/6651 (G to A), as well as the homozygotes A10V, A82T and A245P, will be 10, 2, 5.8, 5.8, 29, 7.6, and 35.4%, respectively, as measured in intact cells after a 1 hour incubation period (Table 8.1, Figure 8.6) (Moisan *et al.*, 1999). On the other hand, knowing that all heterozygote carriers of a deleterious mutation in the HSD3B2 gene are asymptomatic, such a relatively high activity of L173R, i.e., 52.8% was unexpected (Table 8.1), although the apparent instability of the L173R mutant protein would most likely be involved in further reducing the activity catalyzed by the mutant type II 3 $\beta$ -HSD protein in the cells of the adrenals and gonads in patients 46 and 47. It is also possible that the apparent instability of A245P (patient 49) and G294V (patients 33 and 34) will play a role in further decreasing the activity in these patients, as also suggested by their  $V_{\max}/K_m$  values (Figure 8.7). Although the results described above illustrate the almost perfect genotype-phenotype relationship associated with this disorder, there are several examples supporting the notion that there are exceptions to every rule. Firstly, the homozygous A82T mutation which was previously reported in the four Brazilian patients 40, 41, 42 and 43 (Mendonca *et*

*al.*, 1987, 1994). In family 33, the homozygous A82T mutation was associated with precocious puberty, whereas in the unrelated family 32 it was found to be associated with male pseudohermaphroditism, although it had no effect in the homozygous female relative. However, it has recently been demonstrated that although the 46XY individual bearing the A10E homozygous mutation, patient 2, displayed the typical phenotype of ambiguous genitalia at birth and normal virilization at puberty, the female patient 1 also harboring this homozygous mutation underwent spontaneous feminization and menarche (Alos *et al.*, 2000). This is in contrast to the 46XX patient 7 with severe 3 $\beta$ -HSD deficiency (due to a homozygous W171X mutation) who was hypogonadal (Zachmann *et al.*, 1979; Rheaume *et al.*, 1992). As recently indicated by Van Vliet, these latter findings demonstrate the complex relationships between the genotype and the gonadal phenotype in severe 3 $\beta$ -HSD deficiency and the difficulty in predicting fertility (Alos *et al.*, 2000). Another good example is the observation that the Brazilian patients 51 and 52 bearing the homozygous mutation T259M suffer from a non salt-wasting form of the disease (Marui *et al.*, 2000), whereas the homozygous Taiwanese patient 16 also bearing the T259M mutation, the compound heterozygous French patients 19 and 20 with the heterozygous T259M/ 867delG mutation and the homozygous Japanese patients 21 and 22 bearing the T259R mutation were all found to be affected by the severe salt-wasting form of the disease. Moreover, the current data strongly indicate instability of the T259M mutant protein (Moisan *et al.*, 1999; Zhang *et al.*, 2000). Nevertheless, as illustrated in Figure 8.6, very low residual 3 $\beta$ -HSD activity could be observed following a longer incubation period in intact cells expressing the mutant T259M protein when using DHEA as the substrate (Moisan *et al.*, 1999), whereas little activity was evident when using PREG as substrate (Zhang *et al.*, 2000). It can be speculated that the Brazilian patients possess a different steroid responsiveness, but additional experiments would be needed to better understand this apparent discrepancy. It was also unexpected that the L236S mutation found in the compound heterozygous non salt-losing patient 48 (L236S/ 867delG) possesses the same enzyme activity as the wild-type enzyme, with no evidence suggesting that this mutation affects the stability of the protein. Nevertheless, this hypothesis is difficult to reconcile with the well established fact that heterozygous carriers are asymptomatic (Russell *et al.*, 1994; Simard *et al.*, 1995, 1996; Morel *et al.*, 1997). On the other hand, the possibility cannot be ruled out that the L236S mutation could be in linkage disequilibrium with another deleterious mutation affecting the expression or the splicing of this gene.

### Structure-function relationships

Finally, the functional characterization of these mutant enzymes also generated valuable information concerning the structure-function relationships of the 3 $\beta$ -HSD superfamily. Indeed, as indicated in Table 8.1, it is of special interest to note that the amino acid residues that are the sites of missense mutations are generally in highly conserved regions in members of the vertebrate 3 $\beta$ -HSD isoenzymes characterized thus far (Figure 8.2). This finding strongly suggests a crucial role of these residues in the catalytic activity of these enzymes. However, although amino acid Pro<sup>186</sup> is also well conserved in the vertebrate 3 $\beta$ -HSD family, it is not conserved in all members; namely rat type III, mouse types IV and V



and hamster type III. These are specific 3-ketoreductases responsible for the conversion of 3-keto-saturated steroids using NADPH as cofactor and do not share this amino acid residue at this position (Simard *et al.*, 1996; Morel *et al.*, 1997). It is also of interest to mention that mutations A10E, A10V and G15D alter an amino acid residue in the highly conserved Gly-X-X-Gly-X-X-Gly region found in all members of the 3 $\beta$ -HSD superfamily (Baker and Blasco, 1992; Rheume *et al.*, 1995; Morel *et al.*, 1997), which is similar to the common Gly-X-Gly-X-X-Gly conserved sequence present in most NAD (H)-binding enzymes (Scrutton *et al.*, 1990; Lesk, 1995). It is of further interest to mention that the striking phenotypic differences observed between the homozygous salt-losing patients 1 and 2 bearing the A10E mutation (Alos *et al.*, 2000) and the non salt-losing patients 38 and 39 bearing the A10V mutation are in accordance with their respective enzymatic properties, as determined in intact cells (Figure 8.6). Such a difference may be the result of both the apparent instability of the A10E protein coupled with the fact that Glu is a negatively charged residue, whereas Val, like Ala, is a non-polar residue.

Moreover, mutations A82T and G294V create a substitution in each of the two predicted membrane-spanning domains (Simard *et al.*, 1991a, 1996). Furthermore, mutation P155L is located in the first of the two characteristic Y-X-X-X-K sequences located in the region from Tyr<sup>154</sup> to Lys<sup>158</sup> and Tyr<sup>269</sup> to Lys<sup>273</sup> found in the active site of short-chain alcohol dehydrogenases (Scrutton *et al.*, 1990; Krozowski, 1992). Affinity labeling of purified human type I 3 $\beta$ -HSD identified two tryptic peptides comprising amino acids Asn<sup>176</sup> to Arg<sup>186</sup> and Gly<sup>251</sup> to Lys<sup>274</sup> that should contain residues involved in the putative substrate-binding domain (Thomas *et al.*, 1993). Thus the exact role of the first YXXXX motif in the 3 $\beta$ -HSD family remains to be confirmed. Finally, recent findings have shown that His<sup>261</sup> is a critical amino acid residue for 3 $\beta$ -HSD activity and Tyr<sup>253</sup> or Tyr<sup>254</sup> participates in the isomerase activity of the human type I 3 $\beta$ -HSD enzyme (Thomas *et al.*, 1998), in addition to evidence indicating that Tyr<sup>253</sup> functions as the general acid (proton donor) in the isomerase reaction (Mason *et al.*, 1998). Consequently, mutations located within this area will inevitably have a major effect upon enzyme activity, as exemplified in the case of deleterious mutations Y253N, Y254D, T259M and T259R.

### Sequence variants in the HSD3B2 gene versus non-classical 3 $\beta$ -HSD deficiency

Although, as previously mentioned, no mutations have been identified in the HSD3B1 and HSD3B2 genes in the patients diagnosed to be suffering from nonclassical 3 $\beta$ -HSD deficiency, several sequence variants have been identified in these individuals (A167V, S213G, K216E and L236S)(Chang *et al.*, 1993a; Nayak *et al.*, 1998). As illustrated in Figure 8.6, in addition to the L236S mutation the heterozygous A167V sequence variant leads to proteins that have similar activity to the native enzyme, while mutant S213G and K216E proteins have only a minor impact on the activity, retaining 58.4% and 58.95% of the activity of the wild-type, respectively (Table 8.1, patients 53–56)(Moisan *et al.*, 1999). It should also be noted that the mother of patient 53 was also a heterozygous A167V carrier, but did not have any symptoms of hyperandrogenism (Nayak *et al.*, 1998). However, as indicated previously all reported heterozygous carriers bearing a deleterious mutation in the

HSD3B2 gene were typically asymptomatic, these results providing additional molecular proof that other genetic or environmental/hormonal influences may contribute to the expression of the observed symptoms (Zerah *et al.*, 1994; Chang *et al.*, 1995; Forest *et al.*, 1995; Mebarki, 1995; Sakkal-Alkaddour *et al.*, 1996; Tajima *et al.*, 1997). Thus, the functional data concerning these sequence variants coupled with the previous studies reporting that no mutations were found in both HSD3B1 and/or HSD3B2 genes in the patients affected by premature pubarche or hyperandrogenism (Chang *et al.*, 1995; Forest *et al.*, 1995; Mebarki, 1995; Sakkal-Alkaddour *et al.*, 1996; Tajima *et al.*, 1997; Moran *et al.*, 1998) strongly support the conclusion that this disorder does not result from a mutant 3 $\beta$ -HSD isoenzyme.

The possibility that inherited mutation (s) could be located farther upstream in the putative promoter region of the HSD3B2 gene, leading to an aberrant level of expression of a normal type II 3 $\beta$ -HSD protein, cannot be refuted. However, the latter hypothesis is markedly weakened by the fact that all patients come from unrelated pedigrees and diverse ethnic origins. On the other hand, because 3 $\beta$ -HSD gene expression and activity are under complex multiple hormonal regulation (Simard *et al.*, 1996; Mason *et al.*, 1997; Feltus *et al.*, 1999; Gingras and Simard, 1999) it cannot be ruled out that at least some forms of non-classical 3 $\beta$ -HSD deficiency result from a genetic or acquired origin acting indirectly on these modulatory parameters. There is also the potential implication of a steroidogenic enzyme different from the known 3 $\beta$ -HSD isoenzymes. Does it possibly involve dysregulation of 17 $\alpha$ -hydroxylase and 17, 20-lyase activities, as has previously been suggested (Barnes *et al.*, 1993)? If so, is this dysregulation of genetic origin? All these hypotheses require further study to gain more understanding of this puzzling but frequent disease.

## CONCLUSION

In summary, this further insight into the molecular basis of 3 $\beta$ -HSD deficiency has highlighted the fact that mutations in the HSD3B2 gene can result in a wide spectrum of molecular repercussions associated with the different phenotypic manifestations of classical 3 $\beta$ -HSD deficiency. The recent study by Moisan and colleagues (Moisan *et al.*, 1999) has also demonstrated that genotype correlates very well with phenotype in most cases studied, with the exception of patients 51 and 52 bearing the homozygous mutation T259M, and further studies will be needed to elucidate the real impact of the locus harboring the L236S mutation. Moreover, the importance of measuring 17-hydroxypregnenolone levels, which always exceeded 100nmol/L in patients suffering from classical 3 $\beta$ -HSD deficiency, has been suggested. The functional characterization of all the missense mutations known to be involved in this disease also provides valuable information concerning the structure-function relationships of the 3 $\beta$ -HSD superfamily. Finally, this study has highlighted the fact that various mutations appear to have a drastic effect upon the stability of the protein, therefore providing molecular evidence of a new mechanism involved in classical 3 $\beta$ -HSD deficiency.

## REFERENCES

- Abbaszade I.G., Arensburg, J., Park, C.H., Kasa-Vubu, J.Z., Orly, J., Payne, A.H. (1997) Isolation of a new mouse  $3\beta$ -hydroxysteroid dehydrogenase isoform,  $3\beta$ -HSD VI, expressed during early pregnancy. *Endocrinology* **138**, 1392–1399.
- Abbaszade, I.G., Clarke, T.R., Park, C.H. and Payne, A.H. (1995) The mouse  $3\beta$ -hydroxy steroid dehydrogenase multigene family includes two functionally distinct groups of proteins. *Mol. Endocrinol.* **9**, 1214–1222.
- Albrecht, E.D., Aberdeen, G.W., Babischkin, J.S., Tilly, J.L. and Pepe, G.J. (1996) Biphasic developmental expression of adrenocorticotropin receptor messenger ribonucleic acid levels in the baboon fetal adrenal gland. *Endocrinology* **137**, 1292–1298.
- Alos, N., Moisan, A.M., Ward, L., Desrochers, M., Legault, L., Leboeuf, G., *et al.* (2000) A novel A10E homozygous mutation in the HSD3B2 gene causing severe salt-wasting  $3\beta$ -hydroxysteroid dehydrogenase deficiency in 46XX and 46XY French Canadians: evaluation of gonadal function after puberty. *J. Clin. Endocrinol. Metab.* **85**, 1968–1974.
- August, G.P., Grumbach, M.M. and Kaplan, S.L. (1972) Hormonal changes in puberty. III. Correlation of plasma testosterone, LH, FSH, testicular size, and bone age with male pubertal development. *J. Clin. Endocrinol. Metab.* **34**, 319–326.
- Baillie, A.H., Niemi, M. and Ikanen, M. (1965)  $3\beta$ -hydroxysteroid dehydrogenase activity in the human foetal testis. *Acta Endocrinol.* **48**, 429–438.
- Bain, P.A., Meisler, M.H., Taylor, B.A. and Payne, A.H. (1993) The genes encoding gonadal and nongonadal forms of  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase are closely linked on mouse chromosome 3. *Genomics* **16**, 219–223.
- Bain, P.A., Yoo, M., Clarke, T., Hammond, S.H. and Payne, A.H. (1991) Multiple forms of mouse  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase and differential expression in gonads, adrenal glands, liver, and kidneys of both sexes. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8870–8874.
- Baker, M.E. and Blasco, R. (1992) Expansion of the mammalian  $3\beta$ -hydroxy steroid dehydrogenase/ plant dihydroflavonol reductase superfamily to include a bacterial cholesterol dehydrogenase, a bacterial UDP-galactose-4-epimerase, and open reading frames in vaccinia virus and fish lymphocystis disease virus. *FEBS Lett.* **301**, 89–93.
- Barnes, R.B., Ehrmann, D.A., Brigell, D.F. and Rosenfield, R.L. (1993) Ovarian steroidogenic responses to gonadotropin-releasing hormone agonist testing with nafarelin in hirsute women with adrenal responses to adrenocorticotropin suggestive of  $3\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenase deficiency. *J. Clin. Endocrinol. Metab.* **76**, 450–455.
- Barnes, R.B., Rosenfield, R.L., Burstein, S. and Ehrmann, D.A. (1989) Pituitary-ovarian responses to nafarelin testing in the polycystic ovary syndrome. *N. Engl. J. Med.* **320**, 559–565.
- Berube, D., Luu-The, V., Lachance, Y., Gagne, R. and Labrie, F. (1989) Assignment of the human  $3\beta$ -hydroxysteroid dehydrogenase gene (HSD3B) to the P13 band of chromosome 1. *Cytogenet. Cell Genet.* **52**, 199–200.
- Bidlingmayer, F., Dorr, H., Eisenmenger, W., Kuhnle, U. and Knorr, D. (1983) Testosterone and androstenedione concentrations in human testis and epididymis during first two years of life. *J. Clin. Endocrinol. Metab.* **57**, 311–315.
- Bois, E., Mornet, E., Chompret, A., Feingold, J., Hochez, J. and Goulet, V. (1985) Congenital adrenal hyperplasia (21-OH) in France. Population genetics. *Arch. Fr. Ped.* **42**, 175–179.
- Bongiovanni, A.M. (1961) Unusual steroid pattern in congenital adrenal hyperplasia: deficiency of  $3\beta$ -hydroxy steroid dehydrogenase. *J. Clin. Endocrinol.* **21**, 860–862.

- Bongiovanni, A.M. (1962) The adrenogenital syndrome with deficiency of 3 $\beta$ -hydroxy dehydrogenase. *J. Clin. Invest.* **41**, 2086–2092.
- Cara, J.F., Moshang Jr., T., Bongiovanni, A.M. and Marx, B.S. (1985) Elevated 17-hydroxyprogesterone and testosterone in a newborn with 3 $\beta$ -hydroxy steroid dehydrogenase deficiency. *N. Engl. J. Med.* **313**, 618–621.
- Chang, Y.T., Kulin, H.E., Garibaldi, L., Suriano, M.J., Bracki, K. and Pang, S. (1993b) Hypothalamicpituitary-gonadal axis function in pubertal male and female siblings with glucocorticoid-treated nonsalt-wasting 3 $\beta$ -hydroxysteroid dehydrogenase deficiency congenital adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **77**, 1251–1257.
- Chang, Y.T., Wang, J., Yang, X. and Pang, S. (1993a) Molecular basis of the type II 3 $\beta$ -hydroxy steroid dehydrogenase (3 $\beta$ -HSD) gene in patients with non-classic (late-onset) 3 $\beta$ -HSD deficiency congenital adrenal hyperplasia (CAH). In: *Proceedings of the 75th Annual Meeting of the Endocrine Society* (Abstract 1384B), Las Vegas, Nevada.
- Chang, Y.T., Zhang, L., Alkaddour, H.S., Mason, J.I., Lin, K., Yang, X., *et al.*, (1995) Absence of molecular defect in the type II 3 $\beta$ -hydroxy steroid dehydrogenase (3 $\beta$ -HSD) gene in premature pubarche children and hirsute female patients with moderately decreased adrenal 3 $\beta$ -HSD activity. *Ped. Res.* **37**, 820–824.
- Cherradi, N., Defaye, G. and Chambaz, E.M. (1993) Dual subcellular localization of the 3 $\beta$ -hydroxysteroid dehydrogenase isomerase: characterization of the mitochondrial enzyme in the bovine adrenal cortex. *J. Steroid Biochem. Molec. Biol.* **46**, 773–779.
- Cherradi, N., Defaye, G. and Chambaz, E.M. (1994) Characterization of the 3 $\beta$ -hydroxysteroid dehydrogenase activity associated with bovine adrenocortical mitochondria. *Endocrinology* **134**, 1358–1364.
- Cherradi, N., Rossier, M.F., Vallotton, M.B., Timberg, R., Friedberg, I., Orly, J., *et al.*, (1997) Submitochondrial distribution of three key steroidogenic proteins (steroidogenic acute regulatory protein and cytochrome P450<sub>sc</sub> and 3 $\beta$ -hydroxy steroid dehydrogenase isomerase enzymes) upon stimulation by intracellular calcium in adrenal glomerulosa cells. *J. Biol. Chem.* **272**, 7899–7907.
- Clarke, T.R., Bain, P.A., Sha, L. and Payne, A.H. (1993a) Enzyme characteristics of two distinct forms of mouse 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase complementary deoxyribonucleic acids expressed in COS-1 cells. *Endocrinology* **132**, 1971–1976.
- Clarke, T.R., Bain, P.A., Greco, T.L. and Payne, A.H. (1993b) A novel mouse kidney 3 $\beta$ -hydroxysteroid dehydrogenase complementary DNA encodes a 3-ketosteroid reductase instead of a 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase. *Mol. Endocrinol.* **7**, 1569–1578.
- Conley, A.J. and Bird, I.M. (1997) The role of cytochrome P450 17 $\alpha$ -hydroxylase and 3 $\beta$ -hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the  $\Delta^5$  and  $\Delta^4$  pathways of steroidogenesis in mammals. *Biol. Reprod.* **56**, 789–799.
- Couet, J., Simard, J., Martel, C., Trudel, C., Labrie, Y. and Labrie, F. (1992) Regulation of 3-ketosteroid reductase messenger ribonucleic acid levels and 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase activity in rat liver by sex steroids and pituitary hormones. *Endocrinology* **131**, 3034–3044.
- Coulter, C.L., Goldsmith, P.C., Mesiano, S., Voytek, C.C., Martin, M.C., Mason, J.I. and Jaffe, R.B. (1996) Functional maturation of the primate fetal adrenal *in vivo*. II. Ontogeny of corticosteroid synthesis is dependent upon specific zonal expression of 3 $\beta$ -hydroxy steroid dehydrogenase/ isomerase. *Endocrinology* **137**, 4953–4959.
- Cutler, G.B., Bush, G.M., Hodgen, G.D., Graham, C.E. and Loriaux, D.L. (1978) Adrenarche: a survey of rodents, domestic animals and primates. *Endocrinology* **103**, 2112–2118.

- Dardis, A., Saraco, N., Rivarola, M.A. and Belgorosky, A. (1999) Decrease in the expression of the  $3\beta$ -hydroxy steroid dehydrogenase gene in human adrenal tissue during prepuberty and early puberty: implications for the mechanism of adrenarche. *Ped. Res.* **45**, 384–388.
- de Launoit, Y., Zhao, H.F., Belanger, A., Labrie, F. and Simard, J. (1992) Expression of liver-specific member of the  $3\beta$ -hydroxy steroid dehydrogenase family, an isoform possessing an almost exclusive 3- ketosteroid reductase activity. *J. Biol. Chem.* **267**, 4513–4517.
- de Peretti, E. and Forest, M.G. (1982) Pitfalls in the etiological diagnosis of congenital adrenal hyperplasia in the early neonatal period. *Horm. Res.* **16**, 10–22.
- de Peretti, E., Forest, M.G., Feit, J.P. and David, M. (1980) Endocrine studies in two children with male pseudohermaphroditism due to  $3\beta$ -hydroxy steroid dehydrogenase ( $3\beta$ -HSD) defect. In: *Adrenal Androgens*, Genazzani, A.R., Thijssen, J.H.H. and Siiteri, P.K. (eds), Raven Press, New York, pp. 141–145.
- de Peretti, E. and Forest, M.G. (1978) Pattern of plasma dehydroepiandrosterone sulfate levels in human from birth to adulthood. Evidence for testicular production. *J. Clin. Endocrinol. Metab.* **47**, 572–577.
- Dickerman, Z., Grant, D.R., Faiman, C. and Winter, J.S.D. (1984) Intra-adrenal steroid concentrations in man: zonal differences and developmental changes. *J. Clin. Endocrinol. Metab.* **59**, 1031–1036.
- Donohoue, P., Parker, K. and Migeon, C. (1995) Congenital adrenal hyperplasia. In: *The Metabolic and Molecular Basis of Inherited Disease*, Scriver, C.B.A., Sly, W. and Valle, D. (eds), McGraw-Hill, Inc., New York, pp. 2929–2966.
- Doody, K.J., Lorence, M.C., Mason, J.I. and Simpson, E.R. (1990a) Expression of messenger ribonucleic acid species encoding steroidogenic enzymes in human follicles and corpora lutea throughout the menstrual cycle. *J. Clin. Endocrinol. Metab.* **70**, 1041–1045.
- Doody, K.M., Carr, B.R., Rainey, W.E., Byrd, W., Murry, B.A., Strickler, R.C., *et al.* (1990b)  $3\beta$ -hydroxy steroid dehydrogenase/isomerase in the fetal zone and neocortex of the human fetal adrenal gland. *Endocrinology* **126**, 2487–2492.
- Dufort, I., Rheault, P., Huang, X.F., Soucy, P. and Luu-The, V. (1999) Characteristics of a highly labile human type 5  $17\beta$ -hydroxysteroid dehydrogenase. *Endocrinology* **140**, 568–574.
- Dumont, M., Luu-The, V., Dupont, E., Pelletier, G. and Labrie, F. (1992) Characterization, expression, and immunohistochemical localization of  $3\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in human skin. *J. Invest. Derm.* **99**, 415–421.
- Duncan, W.C., Cowen, G.M. and Illingworth, P.J. (1999) Steroidogenic enzyme expression in human corpora lutea in the presence and absence of exogenous human chorionic gonadotrophin (HCG). *Mol. Hum. Reprod.* **5**, 291–298.
- Dupont, E., Labrie, F., Luu-The, V. and Pelletier, G. (1992) Immunocytochemical localization of  $3\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in human ovary. *J. Clin. Endocrinol. Metab.* **74**, 994–998.
- Dupont, E., Luu-The, V., Labrie, F. and Pelletier, G. (1990) Ontogeny of  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase ( $3\beta$ -HSD) in human adrenal gland performed by immunocytochemistry. *Mol. Cell. Endocrinol.* **74**, R7-R10.
- Dupont, E., Luu-The, V., Labrie, F. and Pelletier, G. (1991) Ontogeny of  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase ( $3\beta$ -HSD) in human testis as studied by immunocytochemistry. *J. Androl.* **12**, 161–164.
- Durocher, F., Dumas, C. and Samson, C. (1993) Structure of rat  $3\beta$ -hydroxysteroid dehydrogenase/ 5-ene-4-ene isomerase genes. In: *Proceedings of the 75th Annual Meeting of the Endocrine Society* (Abstract 1530), Las Vegas, Nevada.

- Eldar-Geva, T., Hurwitz, A., Vecsei, P., Paid, Z., Milwidsky, A. and Rosler, A. (1990) Secondary biosynthetic defects in women with late-onset congenital adrenal hyperplasia [see comments]. *N. Engl. J. Med.* **323**, 855–863.
- Faletto, M.B., Linko, P. and Goldstein, J.A. (1992) A single amino acid mutation (Ser<sup>180</sup>-Cys) determines the polymorphism in cytochrome P450g (P450C13) by altering protein stability. *J. Biol. Chem.* **267**, 2032–2037.
- Feinberg, R. and Cohen, R.B. (1965) A comparative histochemical study of the ovarian stromal lipid band, stromal theca cells and normal ovarian follicular apparatus. *Am. J. Obstet. Gynec.* **92**, 958–969.
- Feltus, F.A., Groner, B. and Melner, M.H. (1999) Stat5-mediated regulation of the human type II 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase gene: activation by prolactin. *Mol. Endocrinol.* **13**, 1084–1093.
- Filling, C., Marschall, H.U., Prozorovski, T., Nordling, E., Persson, B., Jornvall, H. and Oppermann, U.C. (1999) Structure-function relationships of 3 $\beta$ -hydroxy steroid dehydrogenases involved in bile acid metabolism. *Adv. Exp. Med. Biol.* **463**, 389–394.
- Fisher, R., Eades, S. and Taylor, N. (1988) Two brothers with congenital adrenal hyperplasia due to partial 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *Ann. Clin. Biochem.* **25** (suppl.), 133–135.
- Forest, M. and Cathiard, A. (1975) Pattern of plasma testosterone and  $\Delta^4$ -androstenedione in normal newborns: evidence for testicular activity at birth. *J. Clin. Endocrinol. Metab.* **41**, 977–984.
- Forest, M.G. (1995) Diagnosis and Treatment of Disorders of Sexual Development. In: *Endocrinology*, L.J. Degroot, (ed), W.B. Saunders & Company, Montreal, pp. 1901–1937.
- Forest, M.G., Mebarki, F., David, A., Bureau, L. and Morel, Y. (1995) Diagnosis of partial 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) questioned: lessons from long-term study and molecular biology. *Horm. Res.* **44**, 55.
- Furster, C. (1999) Hepatic and extrahepatic dehydrogenation/isomerization of 5-cholestene-3 $\beta$ -, 7 $\alpha$ -diol: localization of 3 $\beta$ -hydroxy- $\Delta^5$ -C27-steroid dehydrogenase in pig tissues and subcellular fractions. *Biochim. Biophys. Acta* **1436**, 343–353.
- Furster, C., Zhang, J. and Toll, A. (1996) Purification of a 3 $\beta$ -hydroxy- $\Delta^5$ -C27-steroid dehydrogenase from pig liver microsomes active in major and alternative pathways of bile acid biosynthesis. *J. Biol. Chem.* **271**, 20903–20907.
- Cell, J.S., Carr, B.R., Sasano, H., Atkins, B., Margraf, L., Mason, J.I. and Rainey, W.E. (1998) Adrenarche results from development of a 3 $\beta$ -hydroxy steroid dehydrogenase-deficient adrenal reticularis. *J. Clin. Endocrinol. Metab.* **83**, 3695–3701.
- Gendrel, D., Chaussain, J.L., Roger, M. and Job, J.C. (1979) Congenital adrenal hyperplasia due to blockade of 3 $\beta$ -hydroxysteroid dehydrogenase. *Arch. Fr. Ped.* **36**, 647–655.
- George, F.W. and Wilson, J.D. (1979) The regulation of androgen and estrogen formation in fetal gonads. *Ann. Biol. Animale, Biochim., Biophys.* **19**, 1297–1306.
- Gingras, S. and Simard, J. (1999) Induction of 3 $\beta$ -hydroxysteroid dehydrogenase/isomerase type 1 expression by interleukin-4 in human normal prostate epithelial cells, immortalized keratinocytes, colon, and cervix cancer cell lines. *Endocrinology* **140**, 4573–4584.
- Gingras, S., Moriggl, R., Groner, B. and Simard, J. (1999) Induction of 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase type 1 gene transcription in human breast cancer cell lines and in normal mammary epithelial cells by interleukin-4 and interleukin-13. *Mol. Endocrinol.* **13**, 66–81.
- Goldman, A.S., Yakovac, W.C. and Bongiovanni, A.M. (1966) Development of activity of 3 $\beta$ -hydroxysteroid dehydrogenase in human fetal tissues and in two anencephalic newborns. *J. Clin. Endocrinol. Metab.* **26**, 14–22.

- Grumbach, M.M. and Conte, F.A. (1999) Disorders of sex differentiation. In: *Williams Textbook of Endocrinology*, Wilson, J.D. and Foster, D.W. (eds), Saunders, pp. 1303–1425.
- Hasegawa, T., Ishida, M., Harigaya, T., Ishida, N. and Mukoyama, H. (1998) Molecular cloning, nucleotide sequence and tissue distribution of equine testicular 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase messenger ribonucleic acid. *J. Equine Sci.* **9**, 45–52.
- Hawes, C.S., McBride, M.W., Petropoulos, A., Mueller, U.W. and Sutcliffe, R.G. (1994) Epitopic heterogeneity of human 3 $\beta$ -hydroxy steroid dehydrogenase in villous and extravillous human trophoblast. *J. Mol. Endocrinol.* **12**, 273–281.
- Heinrich, U.E., Bettendorf, M. and Vecsei, P. (1993) Male pseudohermaphroditism caused by non salt-losing congenital adrenal hyperplasia due to 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) deficiency. *J. Steroid Biochem. Molec. Biol.* **45**, 83–85.
- Herman-Giddens, M.E., Slora, E.J., Wasserman, R.C., *et al.*, (1997) Secondary sexual characteristics and menses in young girls seen in office practice: A study from the pediatric research in office settings network. *Pediatrics* **99**, 505–512.
- Janne, O., Perheentupa, J. and Vihko, R. (1970) Plasma and urinary steroids in an eight-year-old boy with 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *J. Clin. Endocrinol. Metab.* **31**, 162–165.
- Jones, G.E.S., Goldberg, B. and Woodruff, J.D. (1968) Histochemistry as a guide for interpretation of cell function. *Am. J. Obstet. Gynec.* **100**, 76–83.
- Kaplan, S. and Grumbach, M. (1978) Pituitary and placental gonadotropin and sex steroids in the human and sub-human primate fetus. *Clin. Endocrinol. Metab.* **7**, 487–511.
- Katsumata, N., Tanae, A., Yasunaga, T., Horikawa, R., Tanaka, T. and Hibi, I. (1995) A novel missense mutation in the type II 3 $\beta$ -hydroxy steroid dehydrogenase gene in a family with classical salt-wasting congenital adrenal hyperplasia due to 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *Hum. Mol. Genet.* **4**, 745–746.
- Keeney, D.S., Naville, D., Milewich, L., Bartke, A. and Mason, J.I. (1993) Multiple isoforms of 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in mouse tissues: male-specific isoforms are expressed in the gonads and liver. *Endocrinology* **133**, 39–45.
- Kenny, F.M., Reynolds, J.W. and Green, O.C. (1971) Partial 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) deficiency in a family with congenital adrenal hyperplasia: evidence for increasing 3 $\beta$ -HSD activity with age. *Pediatrics* **48**, 756–765.
- Krozowski, Z. (1992) 11 $\beta$ -hydroxysteroid dehydrogenase and the short-chain alcohol dehydrogenase (SCAD) superfamily. *Mol. Cell. Endocrinol.* **84**, C25–C31.
- Labrie, F. (1991) Intracrinology. *Mol. Cell. Endocrinol.* **78**, C113–C118.
- Labrie, F., Belanger, A., Cusan, L. and Candas, B. (1997a) Physiological changes in dehydroepiandrosterone are not reflected by serum levels of active androgens and estrogens but of their metabolites: intracrinology. *J. Clin. Endocrinol. Metab.* **82**, 2403–2409.
- Labrie, F., Belanger, A., Cusan, L., Gomez, J.L. and Candas, B. (1997b) Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. *J. Clin. Endocrinol. Metab.* **82**, 2396–2402.
- Labrie, F., Belanger, A., Luu-The, V., *et al.* (1999) Role of DHEA transformation into androgens and estrogens in peripheral intracrine tissues. In: *DHEA: A Comprehensive Review*, Thijssen, J. and Nieuwenhuyse, H. (eds), Parthenon Publishing, New York, pp. 63–103.
- Lachance, Y., Luu-The, V., Labrie, C., Simard, J., Dumont, M., de Launoit, Y., *et al.* (1990) Characterization of human 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase gene and its expression in mammalian cells [published erratum appears in *J. Biol. Chem.* 1992 Feb 15;267 (5): 3551]. *J. Biol. Chem.* **265**, 20469–20475.

- Lachance, Y., Luu-The, V., Verreault, H., Dumont, M., Rheau, E., Leblanc, G. and Labrie, F. (1991) Structure of the human type II 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase (3 $\beta$ -HSD) gene: adrenal and gonadal specificity. *DNA Cell Biol.* **10**, 701–711.
- Lafont, M. (1984) PhD Thesis: le deficit en 3 $\beta$ -ol-deshydrogénase chez l'enfant. Université Claude-Bernard-Lyon I, Lyon, France.
- Lesk, A.M. (1995) NAD-binding domains of dehydrogenases. *Curr. Opin. Struct. Biol.* **5**, 775–783.
- Liu, X.Y., Dangel, A.W., Kelley, R.I., Zhao, W., Denny, P., Botcherby, M., *et al.* (1999) The gene mutated in bare patches and striated mice encodes a novel 3 $\beta$ -hydroxysteroid dehydrogenase. *Nat. Genet.* **22**, 182–187.
- Lorence, M.C., Murry, B.A., Trant, J.M. and Mason, J.I. (1990a) Human 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase from placenta: expression in nonsteroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids. *Endocrinology* **126**, 2493–2498.
- Lorence, M.C., Corbin, C.J., Kamimura, N., Mahendroo, M.S. and Mason, J.I. (1990b) Structural analysis of the gene encoding human 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase. *Mol. Endocrinol.* **4**, 1850–1855.
- Lorence, M.C., Naville, D., Graham-Lorence, S.E., Mack, S.O., Murry, B.A., Trant, J.M. and Mason, J.I. (1991) 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase expression in rat and characterization of the testis isoform. *Mol. Cell. Endocrinol.* **80**, 21–31.
- Luu-The, V., Coté, J. and Labrie, F. (1988) Purification and characterization of human placental 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase. *Clinical Investigations in Medicine*, **11** (suppl.), C32 (Abstract).
- Luu-The, V., Coté, J. and Labrie, F. (1990) Purification of microsomal 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase from human placenta. *Ann. N.Y. Acad. Sci.* **595**, 386–388.
- Luu-The, V., Lachance, Y., Leblanc, G., and Labrie, F. (1992) Human 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase: characterization of three additional related genes. In: *Proceedings of the 74th Endocrine Society Meeting*, pp. 426.
- Luu-The, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, J., Strickler, R. and Labrie, F. (1989) Full length cDNA structure and deduced amino acid sequence of human 3 $\beta$ -hydroxy-5-ene steroid dehydrogenase. *Mol. Endocrinol.* **3**, 1310–1312.
- Luu-The, V., Takahashi, M., de Launoit, Y., Dumont, M., Lachance, Y. and Labrie, F. (1991) Evidence for distinct dehydrogenase and isomerase sites within a single 3 $\beta$ -hydroxysteroid dehydrogenase/5-ene-4-ene isomerase protein. *Biochemistry* **30**, 8861–8865.
- Mapes, S., Corbin, C.J., Tarantal, A. and Conley, A. (1999) The primate adrenal zona reticularis is defined by expression of cytochrome b<sub>5</sub>, 17 $\alpha$ -hydroxylase/17,20-lyase cytochrome P450 (P450c17) and NADPH-cytochrome P450 reductase (reductase) but not 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase (3 $\beta$ -HSD). *J. Clin. Endocrinol. Metab.* **84**, 3382–3385.
- Martel, C., Melner, M.H., Gagne, D., Simard, J. and Labrie, F. (1994) Widespread tissue distribution of steroid sulfatase, 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase (3 $\beta$ -HSD), 17 $\beta$ -HSD, 5 $\alpha$ -reductase and aromatase activities in the rhesus monkey. *Mol. Cell. Endocrinol.* **104**, 103–111.
- Marui, S., Castro, M., Latronico, A.C., Elias, L.L., Arnholt, I.J.P., Moreira, A.C. and Mendoca, B.B. (2000) Mutations in type II 3 $\beta$ -hydroxysteroid dehydrogenase (HSD3B2) gene can cause premature pubarche in girls. *Clin. Endocrinol.* **52**, 67–75.
- Marui, S., Torrealba, I.M., Russell, A.J., Latronico, A.C., Sutcliffe, R.G. and Mendonca, B.B. (1998a) A novel homozygous nonsense mutations E135\* in the type II 3 $\beta$ -hydroxysteroid dehydrogenase gene in a girl with salt-losing congenital adrenal hyperplasia. Mutations in brief no. 168. Online. *Hum. Mutat.* **12**, 139.



- Marui, S., Latronico, A., Arnhold, I., Castro, M., Elias, L., Moreira, A., *et al.*, (1998b) Mutations in the type II 3 $\beta$ -hydroxysteroid dehydrogenase gene in girls with premature pubarche. In: *Proceedings of the 80th Annual meeting of the Endocrine Society*, New Orleans, Louisiana, P2–157.
- Mason, J.I., Keeney, D.S., Bird, I.M., Rainey, W.E., Morohashi, K., Leers-Sucheta, S. and Melner, M.H. (1997) The regulation of 3 $\beta$ -hydroxy steroid dehydrogenase expression. *Steroids* **62**, 164–168.
- Mason, J.I., Naville, D., Evans, B.W. and Thomas, J.L. (1998) Functional activity of 3 P-hydroxy steroid dehydrogenase/isomerase. *Endocr. Res.* **24**, 549–557.
- Mason, J.I., Ushijima, K., Doody, K.M., Nagai, K., Naville, D., Head, J.R., *et al.*, (1993) Regulation of expression of the 3 $\beta$ -hydroxysteroid dehydrogenases of human placenta and fetal adrenal. *J. Steroid Biochem. Molec. Biol.* **47**, 151–159.
- McBride, M.W., McVie, A.J., Burridge, S.M., Brintnell, B., Craig, N., Wallace, A.M., *et al.*, (1999) Cloning, expression, and physical mapping of the 3 $\beta$ -hydroxysteroid dehydrogenase gene cluster (HSD3BP1-HSD3BP5) in humans. *Genomics* **61**, 277–284.
- McCartin, S., Russell, A.J., Fisher, R.A., Wallace, A.M., Arnhold, I.J., Mason, J.I., *et al.*, (2000) Phenotypic variability and origins of mutations in the gene encoding 3 $\beta$ -hydroxysteroid dehydrogenase type II. *J. Mol. Endocrinol.* **24**, 75–82.
- Mebarki, F. (1995) PhD Thesis: Etude moléculaire de causes de pseudohermaphrodisme masculin le syndrome d'insensibilité aux androgènes, le déficit en enzyme de la stéroidogénèse 3 $\beta$ -HSD. Université Lyon, Lyon, France.
- Mebarki, F., Sanchez, R., Rheume, E., Laflamme, N., Simard, J., Forest, M.G., *et al.*, (1995) Nonsalt-losing male pseudohermaphroditism due to the novel homozygous N100S mutation in the type II 3 $\beta$ -hydroxy steroid dehydrogenase gene. *J. Clin. Endocrinol. Metab.* **80**, 2127–2134.
- Medina, M., Herrera, J., Flores, M., Martin, O., Bermudez, J.A. and Zarate, A. (1986) Normal ovarian function in a mild form of late-onset 3 $\beta$ -hydroxy steroid dehydrogenase deficiency. *Fertil. Steril.* **46**, 1021–1025.
- Mendonca, B.B., Bloise, W., Arnhold, I.J., Batista, M.C., Toledo, S.P., Drummond, M.C., *et al.*, (1987) Male pseudohermaphroditism due to nonsalt-losing 3 $\beta$ -hydroxysteroid dehydrogenase deficiency: gender role change and absence of gynecomastia at puberty. *J. Steroid Biochem.* **28**, 669–675.
- Mendonca, B.B., Russell, A.J., Vasconcelos-Leite, M., Arnhold, I.J., Bloise, W., Wajchenberg, B.L., *et al.*, (1994) Mutation in 3 $\beta$ -hydroxy steroid dehydrogenase type II associated with pseudohermaphroditism in males and premature pubarche or cryptic expression in females. *J. Mol. Endocrinol.* **12**, 119–122.
- Mesiano, S., Coulter, C.L. and Jaffe, R.B. (1993) Localization of cytochrome P450 cholesterol side-chain cleavage, cytochrome P450 17 $\alpha$ -hydroxylase/17, 20-lyase and 3 $\beta$ -hydroxysteroid dehydrogenase isomerase steroidogenic enzymes in human and rhesus monkey fetal adrenal glands: reappraisal of functional zonation. *J. Clin. Endocrinol. Metab.* **77**, 1184–1189.
- Milewich, L., Shaw, C.E., Doody, K.M., Rainey, W.E., Mason, J.I. and Carr, B.R. (1991) 3 $\beta$ -Hydroxysteroid dehydrogenase activity in glandular and extraglandular human fetal tissues. *J. Clin. Endocrinol. Metab.* **73**, 1134–1140.
- Miller, W. (1997) The regulation of 17,20 lyase activity. *Steroids* **62**, 133–142.
- Moisan, A.M., Ricketts, M.L., Tardy, V., Desrochers, M., Mebarki, F., Chaussain, J.L., *et al.* (1999) New insight into the molecular basis of 3 $\beta$ -hydroxysteroid dehydrogenase deficiency: identification of eight mutations in the HSD3B2 gene eleven patients from seven new families and comparison of the functional properties of twenty-five mutant enzymes. *J. Clin. Endocrinol. Metab.* **84**, 4410–4425.

- Moran, C., Knochenhauer, E.S. and Azziz, R. (1998) Non-classic adrenal hyperplasia in hyperandrogenism: a reappraisal. *J. Endocrinol. Invest.* **21**, 707–720.
- Morel, Y., Mebarki, F., Rheume, E., Sanchez, R., Forest, M.G. and Simard, J. (1997) Structure/function relationships of 3 $\beta$ -hydroxysteroid dehydrogenase: contribution made by the molecular genetics of 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *Steroids* **62**, 176–184.
- Morissette, J., Rheume, E., Leblanc, J.F., Luu-The, V., Labrie, F. and Simard, J. (1995) Genetic linkage mapping of HSD3B1 and HSD3B2 encoding human types I and II 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase close to D1S514 and the centromeric D1Z5 locus. *Cytogenet. Cell Genet.* **69**, 59–62.
- Nahoul, K., Perrin, C., Leymarie, P. and Job, J.C. (1989) Elevated levels of plasma 4-ene steroids in a case of congenital deficiency of 3 $\beta$ -hydroxysteroid dehydrogenase. *Ann. Endocrinol.* **50**, 58–63.
- Nakabayashi, O., Nomura, O., Nishimori, K. and Mizuno, S. (1995) The cDNA cloning and transient expression of a chicken gene encoding a 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase unique to major steroidogenic tissues. *Gene* **162**, 261–265.
- Nayak, S., Lee, P.A. and Witchel, S.F. (1998) Variants of the type II 3 $\beta$ -hydroxysteroid dehydrogenase gene in children with premature pubic hair and hyperandrogenic adolescents. *Mol. Genet. Metab.* **64**, 184–192.
- Nickson, D.A., McBride, M.W., Zeinali, S., Hawes, C.S., Petropoulos, A., Mueller, U.W. and Sutcliffe, R.G. (1991) Molecular cloning and expression of human trophoblast antigen FDO16GG and its identification as 3 $\beta$ -hydroxy-5-ene steroid dehydrogenase. *J. Reprod. Fertil.* **93**, 149–156.
- Pang, S. (1997) Congenital adrenal hyperplasia. *Bailliere's Clin. Obstet. Gynaecol.* **11**, 281–306.
- Pang, S. (1998) The molecular and clinical spectrum of 3 $\beta$ -hydroxy steroid dehydrogenase deficiency disorder. *Trends Endocrinol. Metab.* **9**, 82–86.
- Pang, S., Levine, L.S., Stoner, E., Opitz, J.M., Pollack, M.S., Dupont, B. and New, M.I. (1983) Nonsalt-losing congenital adrenal hyperplasia due to 3 $\beta$ -hydroxysteroid dehydrogenase deficiency with normal glomerulosa function. *J. Clin. Endocrinol. Metab.* **56**, 808–818.
- Pang, S.Y., Lerner, A.J., Stoner, E., Levine, L.S., Oberfield, S.E., Engel, I. and New, M.I. (1985) Late-onset adrenal steroid 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. I. A cause of hirsutism in pubertal and postpubertal women. *J. Clin. Endocrinol. Metab.* **60**, 428–439.
- Parker, Jr. C.R., Faye-Petersen, O., Stankovic, A.K., Mason, J.I. and Grizzle, W.E. (1995) Immunohistochemical evaluation of the cellular localization and ontogeny of 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in the human fetal adrenal gland. *Endocr. Res.* **21**, 69–80.
- Parks, G.A., Bermudez, J.A., Anast, C.S., Bongiovanni, A.M. and New, M.I. (1971) Pubertal boy with the 3 $\beta$ -hydroxysteroid dehydrogenase defect. *J. Clin. Endocrinol. Metab.* **33**, 269–278.
- Paula, F.J., Dick-de-Paula, I., Pontes, A., Schmitt, F.C., Mendonca, B.B. and Foss, M.C. (1994) Hyperandrogenism due to 3 $\beta$ -hydroxy steroid dehydrogenase deficiency with accessory adrenocortical tissue: a hormonal and metabolic evaluation. *Braz. J. Med. Biol. Res.* **27**, 1149–1158.
- Payne, A.H., Abbaszade, I.G., Clarke, T.R., Bain, P.A. and Park, C.H. (1997) The multiple murine 3 $\beta$ -hydroxy steroid dehydrogenase isoforms: structure, function, and tissue- and developmentally specific expression. *Steroids* **62**, 169–175.
- Peltoketo, H., Luu-The, V., Simard, J. and Adamski, J. (1999) 17 $\beta$ -hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family: nomenclature and main characteristics of the 17HSD/KSR enzymes. *J. Mol. Endocrinol.* **23**, 1–11.
- Quigley, C.A. (1998) Disorders of sex determination and differentiation. In: *Principles of Molecular Medicine*, Jameson, J.L. (ed), Humana Press, New Jersey, pp. 527–559.

- Rheume, E., Lachance, Y., Zhao, H.F., Breton, N., Dumont, M., de Launoit, Y., *et al.*, (1991) Structure and expression of a new complementary DNA encoding the almost exclusive 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in human adrenals and gonads. *Mol. Endocrinol.* **5**, 1147–1157.
- Rheume, E., Sanchez, R., Mebarki, F., Gagnon, E., Carel, J.C., Chaussain, J.L., *et al.*, (1995) Identification and characterization of the G15D mutation found in a male patient with 3 $\beta$ -hydroxy steroid dehydrogenase (3 $\beta$ -HSD) deficiency: alteration of the putative NAD-binding domain of type II 3 $\beta$ -HSD. *Biochemistry* **34**, 2893–2900.
- Rheume, E., Sanchez, R., Simard, J., Chang, Y.T., Wang, J., Pang, S. and Labrie, F. (1994) Molecular basis of congenital adrenal hyperplasia in two siblings with classical nonsalt-losing 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *J. Clin. Endocrinol. Metab.* **79**, 1012–1018.
- Rheume, E., Simard, J., Morel, Y., Mebarki, F., Zachmann, M., Forest, M.G., *et al.*, (1992) Congenital adrenal hyperplasia due to point mutations in the type II 3 $\beta$ -hydroxysteroid dehydrogenase gene. *Nat. Genet.* **1**, 239–245.
- Ricketts, M.L., Shoesmith, K.J., Hewison, M., Strain, A., Eggo, M.C. and Stewart, P.M. (1998) Regulation of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 in primary cultures of rat and human hepatocytes. *J. Endocrinol.* **156**, 159–168.
- Riley, S.C., Bassett, N.S., Berdusco, E.T., Yang, K., Leystra-Lantz, C., Luu-The, V., *et al.*, (1993) Changes in the abundance of mRNA for type-I 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in the human placenta and fetal membranes during pregnancy and labor. *Gynecol. Obst. Invest.* **35**, 199–203.
- Riley, S.C., Dupont, E., Walton, J.C., Luu-The, V., Labrie, F., Pelletier, G. and Challis, J.R. (1992) Immunohistochemical localization of 3 $\beta$ -hydroxy-5-ene-steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in human placenta and fetal membranes throughout gestation. *J. Clin. Endocrinol. Metab.* **75**, 956–961.
- Rogerson, F.M., Courtemanche, J., Fleury, A., Head, J.R., LeHoux, J.-G. and Mason, J.I. (1998) Characterization of cDNAs encoding isoforms of hamster 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase. *J. Mol. Endocrinol.* **20**, 99–110.
- Rogerson, F.M., LeHoux, J.-G. and Mason, J.I. (1996) Expression and characterization of isoforms of 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in the hamster. *J. Steroid Biochem. Molec. Biol.* **55**, 481–487.
- Rosenfield, R.L., Barmach de Niepmiszshe, A., Kenny, F.M. and Genel, F. (1974) The response to human chorionic gonadotropin (hCG) administration in boys with and without  $\Delta^5$ -3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *J. Clin. Endocrinol. Metab.* **39**, 370–374.
- Rosenfield, R.L., Rich, B.H., Wolfsdorf, J.I., Cassorla, F., Parks, J.S., Bongiovanni, A.M., *et al.*, (1980) Pubertal presentation of congenital  $\Delta^5$ -3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *J. Clin. Endocrinol. Metab.* **51**, 345–353.
- Russell, A., Nazer, H., Shams, A., Sjovall, J. and Sutcliffe, R. (1995) No linkage to the 3 $\beta$ -HSD gene cluster in a kindred affected with 3 $\beta$ -hydroxy- $\Delta^5$ -C27-steroid dehydrogenase deficiency and early onset hepatic failure. *Hum. Genet.* **95**, 586–588.
- Russell, A.J., Wallace, A.M., Forest, M.G., Donaldson, M.D., Edwards, C.R. and Sutcliffe, R.G. (1994) Mutation in the human gene for 3 $\beta$ -hydroxysteroid dehydrogenase type II leading to male pseudohermaphroditism without salt loss. *J. Mol. Endocrinol.* **12**, 225–237.
- Sakai, N., Tanaka, M., Takahashi, M., Fukada, S., Mason, J.I. and Nagahama, Y. (1994) Ovarian 3 P-hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase of rainbow trout: its cDNA cloning and properties of the enzyme expressed in a mammalian cell. *FEBS Lett.* **350**, 309–313.
- Sakkal-Alkaddour, H., Zhang, L., Yang, X., Chang, Y.T., Kappy, M., Slover, R.S., *et al.*, (1996) Studies of 3-hydroxysteroid dehydrogenase genes in infants and children manifesting premature

- pubarche and increased adrenocorticotropin-stimulated  $\Delta^5$ -steroid levels. *J. Clin. Endocrinol. Metab.* **81**, 3961–3965.
- Sanchez, R., de Launoit, Y., Durocher, F., Belanger, A., Labrie, F. and Simard, J. (1994a) Formation and degradation of dihydrotestosterone by recombinant members of the rat 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase family. *Mol. Cell. Endocrinol.* **103**, 29–38.
- Sanchez, R., Mebarki, F., Rheume, E., Laflamme, N., Forest, M.G., Bey-Omar, F., *et al.*, (1994b) Functional characterization of the novel L108W and P186L mutations detected in the type II 3 $\beta$ -hydroxysteroid dehydrogenase gene of a male pseudohermaphrodite with congenital adrenal hyperplasia. *Hum. Mol. Genet.* **3**, 1639–1645.
- Sanchez, R., Rheume, E., Laflamme, N., Rosenfield, R.L., Labrie, F. and Simard, J. (1994c) Detection and functional characterization of the novel missense mutation Y254D in type II 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) gene of a female patient with nonsalt-losing 3 $\beta$ -HSD deficiency. *J. Clin. Endocrinol. Metab.* **78**, 561–567.
- Sasano, H., Mori, T., Sasano, N., Nagura, H. and Mason, J.I. (1990) Immunolocalization of 3 $\beta$ -hydroxy steroid dehydrogenase in human ovary. *J. Reprod. Fertil.* **89**, 743–751.
- Sauer, L.A., Chapman, J.C. and Dauchy, R.T. (1994) Topology of 3 $\beta$ -hydroxy-5-ene-steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase in adrenal cortex mitochondria and microsomes. *Endocrinology* **134**, 751–759.
- Schram, P., Zerah, M., Mani, P., Jewelewicz, R., Jaffe, S. and New, M.I. (1992) Nonclassical 3 $\beta$ -hydroxy steroid dehydrogenase deficiency: a review of our experience with 25 female patients. *Fertil. Steril.* **58**, 129–136.
- Scrutton, N.S., Berry, A. and Perham, R.N. (1990) Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature* **343**, 38–43.
- Seron-Ferre, M., Lawrence, C.C. and Jaffe, R.B. (1978) Steroid production by definitive and fetal zones of the human fetal adrenal gland. *J. Clin. Endocrinol. Metab.* **46**, 603–609.
- Siiteri, P.K. and Wilson, J.D. (1974) Testosterone formation and metabolism during male sexual differentiation in human embryo. *J. Clin. Endocrinol. Metab.* **38**, 113–125.
- Simard, J., Couet, J., Durocher, F., Labrie, Y., Sanchez, R., Breton, N., *et al.* (1993a) Structure and tissue-specific expression of a novel member of the rat 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase (3 $\beta$ -HSD) family. The exclusive 3 $\beta$ -HSD gene expression in the skin. *J. Biol. Chem.* **268**, 19659–19668.
- Simard, J., de Launoit, Y. and Labrie, F. (1991a) Characterization of the structure-activity relationships of rat types I and II 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase by site-directed mutagenesis and expression in HeLa cells. *J. Biol. Chem.* **266**, 14842–14845.
- Simard, J., Durocher, F., Mebarki, F., Turgeon, C., Sanchez, R., Labrie, Y., *et al.*, (1996) Molecular biology and genetics of the 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase gene family. *J. Endocrinol.* **150** (Suppl.), S189–S207.
- Simard, J., Melner, M.H., Breton, N., Low, K.G., Zhao, H.F., Periman, L.M. and Labrie, F. (1991b) Characterization of macaque 3-hydroxy-5-ene steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase: structure and expression in steroidogenic and peripheral tissues in primate. *Mol. Cell. Endocrinol.* **75**, 101–110.
- Simard, J., Rheume, E., Leblanc, J.F., Wallis, S.C., Joplin, G.F., Gilbey, S., *et al.* (1994) Congenital adrenal hyperplasia caused by a novel homozygous frameshift mutation 273  $\Delta\Delta\Delta$  in type II 3 $\beta$ -hydroxysteroid dehydrogenase gene (HSD3B2) in three male patients of Afghan/Pakistani origin. *Hum. Mol. Genet.* **3**, 327–330.
- Simard, J., Rheume, E., Mebarki, F., Sanchez, R., New, M.I., Morel, Y. and Labrie, F. (1995) Molecular basis of human 3 $\beta$ -hydroxy steroid dehydrogenase deficiency. *J. Steroid Biochem. Molec. Biol.* **53**, 127–138.

- Simard, J., Rheume, E., Sanchez, R., Laflamme, N., de Launoit, Y., Luu-The, V., *et al.* (1993b) Molecular basis of congenital adrenal hyperplasia due to  $3\beta$ -hydroxysteroid dehydrogenase deficiency. *Mol. Endocrinol.* **7**, 716–728.
- Simonian, M.H. and Gill, G.N. (1981) Regulation of the fetal human adrenal cortex: effects of adrenocorticotropin on growth and function of monolayer cultures of fetal and definitive zone cells. *Endocrinology* **108**, 1769–1779.
- Sutcliffe, R.G., Russell, A.J., Edwards, C.R. and Wallace, A.M. (1996) Human  $3\beta$ -hydroxy steroid dehydrogenase: genes and phenotypes. *J. Mol. Endocrinol.* **17**, 1–5.
- Suzuki, T., Sasano, H., Kimura, N., Tamura, M., Fukaya, T., Yajima, A. and Nagura, H. (1994) Immunohistochemical distribution of progesterone, androgen and oestrogen receptors in the human ovary during the menstrual cycle: relationship to expression of steroidogenic enzymes. *Hum. Reproduct.* **9**, 1589–1595.
- Suzuki, T., Sasano, H., Tamura, M., Aoki, H., Fukaya, T., Yajima, A., *et al.* (1993) Temporal and spatial localization of steroidogenic enzymes in prerenopausal human ovaries: *In situ* hybridization and immunohistochemical study. *Mol. Cell. Endocrinol.* **97**, 135–143.
- Swofford, D.L. (1993) PAUP: *Phylogenetic Analysis Using Parsimony*, Computer program distributed by Sinauer Associates.
- Tajima, T., Fujieda, K., Nakae, J., Shinohara, N., Yoshimoto, M., Baba, T., *et al.* (1995) Molecular analysis of type II  $3\beta$ -hydroxysteroid dehydrogenase gene in Japanese patients with classical  $3\beta$ -hydroxy steroid dehydrogenase deficiency. *Hum. Mol. Genet.* **4**, 969–971.
- Tajima, T., Nishi, Y., Takase, A., Nakae, J., Murashita, M. and Fujieda, K. (1997) No genetic mutation in type II  $3\beta$ -hydroxysteroid dehydrogenase gene in patients with biochemical evidence of enzyme deficiency. *Horm. Res.* **47**, 49–53.
- Thomas, J.L., Evans, B.W., Blanco, G., Mercer, R.W., Mason, J.I., Adler, S., *et al.* (1998) Site-directed mutagenesis identifies amino acid residues associated with the dehydrogenase and isomerase activities of human type I (placental)  $3\beta$ -hydroxysteroid dehydrogenase/isomerase. *J. Steroid Biochem. Molec. Biol.* **66**, 327–334.
- Thomas, J.L., Evans, B.W. and Strickler, R.C. (1997) Affinity radiolabeling identifies peptides associated with the isomerase activity of human type I (placental)  $3\beta$ -hydroxysteroid dehydrogenase/ isomerase. *Biochemistry* **36**, 9029–9034.
- Thomas, J.L., Frieden, C., Nash, W.E. and Strickler, R.C. (1995) An NADH-induced conformational change that mediates the sequential  $3\beta$ -hydroxysteroid dehydrogenase/isomerase activities is supported by affinity labeling and the time-dependent activation of isomerase. *J. Biol. Chem.* **270**, 21003–21008.
- Thomas, J.L., Myers, R.P., Rosik, L.O. and Strickler, R.C. (1990) Affinity alkylation of human placental  $3\beta$ -hydroxy-5-ene-steroid dehydrogenase and steroid 5-4-ene-isomerase by  $2\alpha$ -bromoacetoxyprogesterone: evidence for separate dehydrogenase and isomerase sites on one protein. *J. Steroid Biochem.* **36**, 117–123.
- Thomas, J.L., Nash, W.E., Crankshaw, M.W. and Strickler, R.C. (1994) Affinity labeling in the presence of the reduced diphosphopyridine nucleotide NADH identifies peptides associated with the activities of human placental  $3\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenase/isomerase. *J. Soc. Gynecol. Invest.* **1**, 155–163.
- Thomas, J.L., Nash, W.E., Myers, R.P., Crankshaw, M.W. and Strickler, R.C. (1993) Affinity radiolabeling identifies peptides and amino acids associated with substrate binding in human placental  $3\beta$ -hydroxy- $\Delta^5$ -steroid dehydrogenase. *J. Biol. Chem.* **268**, 18507–18512.
- Van Seters, A., Degenhart, H., Moolenaar, A.J., Swinkels, L.M.J.M., Waelkens, J.J., *et al.* (1990) Biochemical concealment of  $3\beta$ -HSD deficiency by long term steroid substitution. *J. Steroid Biochem.* **36** (suppl.), 174.

- Vermeulen, A. (1976) The hormonal activity of the postmenopausal ovary. *J. Clin. Endocrinol. Metab.* **42**, 247–253.
- Voutilainen, R., Ilvesmaki, V. and Miettinen, P.J. (1991) Low expression of 3 $\beta$ -hydroxy-5-ene steroid dehydrogenase gene in human fetal adrenals *in vivo*; adrenocorticotropin and protein kinase C-dependent regulation in adrenocortical cultures. *J. Clin. Endocrinol. Metab.* **72**, 761–767.
- Wilson, R. and New, M.I. (1998) Congenital adrenal hyperplasia. In: *Principles of Molecular Medicine*, edited by Jameson, J.L. (ed), Humana Press, New Jersey, pp. 481–493.
- Winter, J., Hughes, I. and Reyes, F. (1976) Pituitary-gonadal relations in infancy. 2: Patterns of serum gonadal steroid concentrations in man from birth to two years of age. *J. Clin. Endocrinol. Metab.* **42**, 679–686.
- Wolthers, B.C., de Vries, I.J., Volmer, M. and Nagel, G.T. (1987) Detection of 3 $\beta$ -hydroxy steroid dehydrogenase deficiency in a newborn by means of urinary steroid analysis. *Clin. Chem. Acta* **169**, 109–116.
- Yoshimoto, M., Baba, T., Yokoo, T., Matsumoto, T., Hayashi, S., Yasui, M., Fukuda, S. and Yanagi, T. (1988) Case of salt-losing congenital adrenal hyperplasia due to 3 $\beta$ -hydroxy steroid dehydrogenase deficiency: the first Japanese patient. *Acta Paed. Jap.* **92**, 1964–1970.
- Yoshimoto, M., Kawaguchi, T., Mori, R., Kinoshita, E., Baba, T., Tajima, T., *et al.* (1997) Pubertal changes in testicular 3 $\beta$ -hydroxy steroid dehydrogenase activity in a male with classical 3 $\beta$ -hydroxysteroid dehydrogenase deficiency showing spontaneous secondary sexual maturation. *Horm. Res.* **48**, 83–87.
- Zachmann, M., Forest, M.G. and De Peretti, E. (1979) 3 $\beta$ -hydroxysteroid dehydrogenase deficiency: follow-up study in a girl with pubertal bone age. *Horm. Res.* **11**, 292–302.
- Zachmann, M., Kempken, B., Anner, I. and Pezzoli, V. (1988) Age-related differences in the excretion of  $\Delta^5$  steroids (D5S) in two related girls with 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *Fed. Res.* **24**, 543.
- Zachmann, M., Vollmin, J.A., Murset, G., Curtius, H.C. and Prader, A. (1970) Unusual type of congenital adrenal hyperplasia probably due to deficiency of 3 $\beta$ -hydroxysteroid dehydrogenase. Case report of a surviving girl and steroid studies. *J. Clin. Endocrinol. Metab.* **30**, 719–726.
- Zerah, M., Rheume, E., Mani, P., Schram, P., Simard, J., Labrie, F. and New, M.I. (1994) No evidence of mutations in the genes for type I and type II 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) in nonclassical 3 $\beta$ -HSD deficiency. *J. Clin. Endocrinol. Metab.* **79**, 1811–1817.
- Zhang, L., Mason, J.I., Naiki, Y., Copeland, K.C., Castro-Magana, M., Gordon-Walker, T.T., Chang, Y.T. and Pang, S. (2000) Characterization of two novel homozygous missense mutations involving codon 6 and 259 of type II 3 $\beta$ -hydroxysteroid dehydrogenase (3BHSD) gene causing, respectively, nonsalt-wasting and salt-wasting 3 $\beta$ HSD deficiency disorder. *J. Clin. Endocrinol. Metab.* **85**, 1678–1685.
- Zhang, L., Sakkal-Alkaddour, H., Chang, Y.T., Yang, X. and Pang, S. (1996) A new compound heterozygous frameshift mutation in the type II 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) gene causes salt-wasting 3 $\beta$ -HSD deficiency congenital adrenal hyperplasia. *J. Clin. Endocrinol. Metab.* **81**, 291–295.
- Zhao, H.F., Labrie, C., Simard, J., de Launoit, Y., Trudel, C., Martel, C., *et al.* (1991) Characterization of rat 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase cDNAs and differential tissue-specific expression of the corresponding mRNAs in steroidogenic and peripheral tissues. *J. Biol. Chem.* **266**, 583–593.
- Zhao, H.F., Rheume, E., Trudel, C., Couet, J., Labrie, F. and Simard, J. (1990) Structure and sexual dimorphic expression of a liver-specific rat 3 $\beta$ -hydroxysteroid dehydrogenase/isomerase. *Endocrinology* **127**, 3237–3239.

- Zhao, H.F., Simard, J., Labrie, C., Breton, N., Rheaume, E., Luu-The, V. and Labrie, F. (1989) Molecular cloning, cDNA structure and predicted amino acid sequence of bovine 3 $\beta$ -hydroxy-5-ene steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase. *FEBS Lett.* **259**, 153–157.

## 9.

# HUMAN 17 $\alpha$ -HYDROXYLASE/17, 20-LYASE

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P450c17 (CYP17) catalyzes both 17 $\alpha$ -hydroxylase and 17,20-lyase activities, and hence is the qualitative regulator of human steroidogenesis, determining which class of steroids will be produced. The two activities are differentially regulated developmentally and in a tissue-specific fashion. Biochemical, computational, and mutagenesis studies all indicate that the ratio of lyase to hydroxylase activity is regulated by the availability of the electron donor, NADPH-cytochrome P450 oxidoreductase, and by the ability of CYP17 to interact with this reductase in a productive fashion. Three factors can increase the lyase:hydroxylase ratio: (1) increasing the molar abundance of P450 oxidoreductase; (2) increasing the abundance of cytochrome b<sub>5</sub>, which allosterically facilitates interaction between P450c17 and the oxidoreductase; and (3) serine/threonine phosphorylation of CYP17 itself. These multiple mechanisms permit the fine control of adrenal and gonadal C19 steroid production.

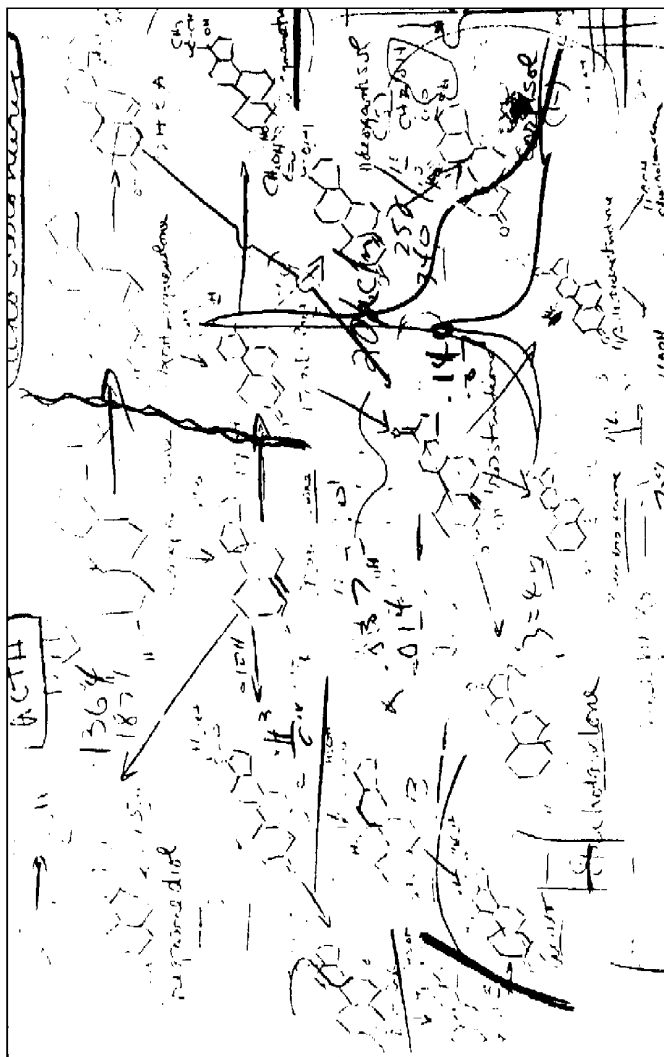
KEY WORDS: adrenarche, CYP17, cytochrome P450c17, cytochrome b<sub>5</sub>, sex steroids.

## HISTORY

The study of human P450c17 (also known as CYP 17) shows how basic and clinical investigation can solve a complex problem only after dogma is rejected and conclusive but unexpected results are reproduced and accepted. By the early 1960s the major steroid hormones had been isolated and characterized chemically, and their biosynthetic pathways were being deduced, but the nature of the various biosynthetic enzymes was unclear. In 1964, Omura and Sato described the hepatic microsomal proteins whose characteristic absorption spectra defined the cytochrome P450 enzymes (Omura and Sato, 1964), and in 1965, Cooper *et al.* published their landmark study of P450-mediated steroid 21-hydroxylation was published (Cooper *et al.*, 1965); yet the reconstitution of the P450 catalytic system with phospholipid, flavoprotein, and NADPH was still years away (Haugen and Coon, 1976). It was clear that a human 17 $\alpha$ -hydroxylase had to exist, but it was not yet known whether this enzyme was a P450 or not.

Thus knowledge was primitive in 1963 when a 35-year old woman with severe hypertension, hypokalemia, primary amenorrhea and absent secondary sexual characteristics was referred to Dr. Ed Biglieri at the San Francisco General Hospital.





**Figure 9.1 Blackboard in the office of Dr. Edward Bilgeri in the General Clinical Research Center at San Francisco General Hospital circa 1965.** Bilgeri *et al.* analyzed dozens of blood and urine specimens for various steroid hormones in their quest to determine why young women had mineralocorticoid-induced hypertension despite undetectable aldosterone production. Their exhaustive probing of the steroid biosynthetic and degradative pathways defined the first case of steroid 17 $\alpha$ -hydroxylase deficiency (Billieri *et al.*, 1966). Photo courtesy of Dr. Edward Biglieri

The presumptive diagnosis of primary aldosteronism was rejected as the patient's urine contained no measurable metabolites of cortisol, aldosterone, or 17-ketosteroids. An intensive investigation that spanned more than three years (Figure 9.1) ascertained that the patient excreted huge amounts of corticosterone metabolites and that the mineralocorticoid responsible for her hypertension was 11-deoxycorticosterone (Biglieri *et al.*, 1966). This steroid excretion profile indicated an inability to 17-hydroxylate steroid hormone precursors and defined the clinical features of 17 $\alpha$ -hydroxylase deficiency. More importantly, her inability to synthesize sex steroids suggested either that the 17 $\alpha$ -hydroxylase enzyme was also the 17,20-lyase enzyme and/or that 17-hydroxysteroids were obligate precursors of the C19 sex steroid precursors—a conundrum that would not be resolved for 20 years.

### ONE ENZYME OR TWO?

In 1972, six years after Biglieri's investigations were published, Zachman and colleagues described a child with 46XY male pseudohermaphroditism who showed normal urinary excretion of 17-hydroxycorticosteroids (17OHCS), increased urinary pregnanetriol (the metabolite of 17 $\alpha$ -hydroxyprogesterone, 17OHP), no response of urinary testosterone to gonadal stimulation with hCG, and a normal response of urinary 17OHCS to ACTH. Furthermore, incubation of biopsied testicular tissue with DHEA or  $\Delta^4$ -androstenedione produced testosterone, but incubation of tissue with pregnenolone, progesterone, 17 $\alpha$ -hydroxypregnenolone and 17OHP produced no testosterone. Thus the patient was identified as having "Steroid 17,20-desmolase deficiency: a new cause of male pseudohermaphroditism" (Zachman *et al.*, 1972). Although the evaluation of the patient's steroidogenesis was truly elegant for its time, the conclusion was hampered by the "one enzyme, one activity" paradigm that dominated biochemistry at the time. Thus, this case led to the logical conclusion that 17 $\alpha$ -hydroxylase and 17,20-lyase were two distinct proteins. This dogma was not challenged for the next decade, and was strengthened by additional reports of isolated 17,20-lyase deficiency (Goebelsmann *et al.*, 1976; Campo *et al.*, 1979; Forest *et al.*, 1980; Zachmann *et al.*, 1982; Larrea *et al.*, 1983; Ratner *et al.*, 1983; de Peretti *et al.*, 1984). However, between 1981 and 1983, Nakajin and Hall and others reported the heretical observation that the 17 $\alpha$ -hydroxylase and 17,20-lyase activities of neonatal porcine testis copurified (Nakajin and Hall, 1981a, b; Nakajin *et al.*, 1981, 1983; Kominami *et al.*, 1982), but the biochemistry and endocrinology communities initially disregarded this work. The addition of cytochrome  $b_5$  to these purified preparations dramatically increased only the 17,20-lyase activity (Onoda and Hall, 1982), and Hall's own protein sequencing data suggested that adrenal and testicular P450c17 were distinct proteins (Nakajin *et al.*, 1984). The controversies were not settled until P450c17 was cloned. Zuber and colleagues reported the cloning of bovine P450c17 cDNA reverse transcribed from an enriched fraction of mRNA that had been size-selected by sucrose gradient centrifugation (Zuber *et al.*, 1986a). Chung and colleagues first purified and micro-sequenced porcine

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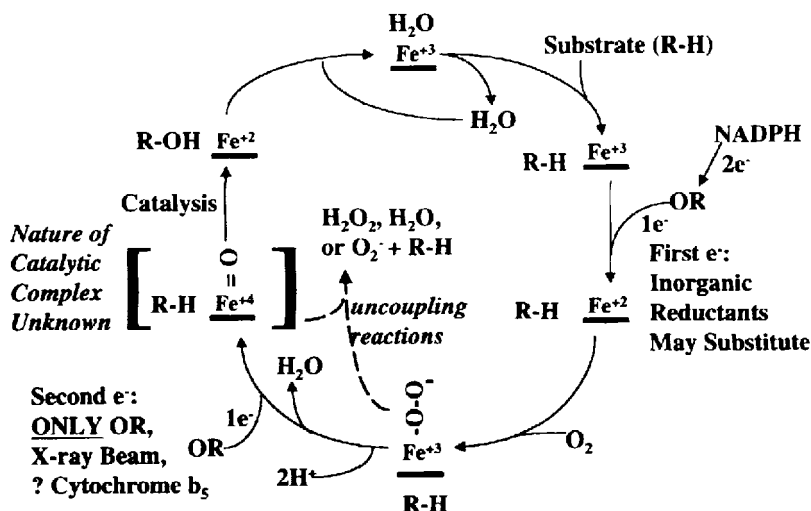
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P450c17, obtained a small porcine cDNA, and used this to clone human adrenal and testicular P450c17 cDNA (Chung *et al.*, 1987). Zuber *et al.* also expressed the bovine cDNA in transfected COS-1 cells, providing that this single protein conferred both 17 $\alpha$ -hydroxylase and 17,20-lyase activities to these non-steroidogenic cells (Zuber *et al.*, 1986a). Thus Zuber's transfection studies and Chung's cloning identical cDNAs from two tissues finally settled the arguments: there is only one CYP17 enzyme that catalyzes both activities in all steroidogenic tissues.

### MECHANISM OF CATALYSIS

P450c17 is a 57kDa protein located in the endoplasmic reticulum, where it receives two electrons from NADPH via the flavoprotein P450 oxidoreductase (OR) during its catalytic cycle (Baron *et al.*, 1972; Yasukochi *et al.*, 1976). Like all microsomal P450s, P450c17 receives these electrons in two discrete one-electron steps, with substrate and molecular oxygen binding between these transfers of electrons (Figure 9.2). By receiving these electrons, P450c17 can catalyze both the 17 $\alpha$ -hydroxylation and 17,20-lyase activity with both  $\Delta^5$  steroids (pregnenolone, 17-hydroxypregnenolone) and A steroids (progesterone, 17-hydroxyprogesterone). However, the relative efficiencies of these four reactions vary based on the nature of the available electron-donating redox partners and also vary depending on amino acid sequence differences in P450c17 from different species. We shall primarily consider the reactions with human P450c17.

Although the catalytic cycle of P450c17 uses the same initial steps whether pregnenolone, progesterone, or their 17 $\alpha$ -hydroxylated derivatives are substrates, P450c17 catalyzes at least three fundamentally distinct chemical transformations. First, the 17 $\alpha$ -hydroxylase reaction is a typical P450-mediated hydroxylation, believed to proceed via an "oxygen rebound" mechanism involving a ferryl oxene as the active oxygenating species (Ortiz de Montellano, 1986). Human P450c17 also 16 $\alpha$ -hydroxylates at least progesterone and dehydroepiandrosterone (DHEA), presumably by the same mechanism (Lin *et al.*, 1993; Swart *et al.*, 1993). Second, human P450c17 converts 17 $\alpha$ -hydroxysteroids to C19 steroids via the 17,20-lyase reaction, in which a carbon—carbon bond is oxidatively cleaved to yield the ketosteroid and acetic acid. Unlike the 17 $\alpha$ -hydroxylase reaction, which is roughly equally efficient for both  $\Delta^5$  and  $\Delta^4$  steroids, the human 17,20-lyase reaction is nearly two orders of magnitude more efficient with the  $\Delta^5$  steroid 17-hydroxypregnenolone than with its  $\Delta^4$  homolog, 17-hydroxyprogesterone (Auchus *et al.*, 1998) although this is not so for rat P450c17. This discrepancy results from the combination of a 10-fold higher  $K_m$  and a 10-fold slower  $V_{max}$  for 17-hydroxyprogesterone compared to 17-hydroxypregnenolone (Lee-Robichaud *et al.*, 1995; Auchus *et al.*, 1998). Kinetic constants obtained for the four principal reactions using His-tagged modified P450c17 purified from *E. coli* (Lee-Robichaud *et al.*, 1995) and native microsomal P450c17 expressed in yeast (Auchus *et al.*, 1998) are remarkably consistent, confirming differences in both affinity and turnover rates for the 17, 20-lyase substrates. Third, in the presence of cytochrome b<sub>5</sub> an alternate pathway of pregnenolone metabolism forms 5,16-androstadien-3 $\beta$ -ol via another carbon-carbon bond cleavage reaction (Lee-Robichaud *et al.*, 1995). In pigs, this third pathway forms an andiene pheromone of some biological importance (Nakajin *et al.*, 1985), but the role of the



**Figure 9.2 The P450 catalytic cycle.** The cycle begins when substrate displaces the axial water molecule bound to the heme iron. Substrate binding lowers the redox potential of the heme sufficiently to allow its reduction first by the transfer of one electron from OR, thus lowering the oxidation state of the heme iron from the ferric (Fe<sup>3+</sup>) to the ferrous (Fe<sup>2+</sup>) state. Dioxygen binds to the Fe<sup>2+</sup> center, followed by two protons. During productive catalytic cycles, the transfer of the second electron generates water and the active oxygenating species, probably a ferryl (Fe<sup>4+</sup>) oxene. A ferryl oxene mechanism is generally accepted for the 17 $\alpha$ -hydroxylase reaction, and we propose a similar mechanism for the 17,20-lyase reaction (see [Figure 9.4](#)). Unproductive cycles generate reduced forms of O<sub>2</sub> such as H<sub>2</sub>O<sub>2</sub> or superoxide (O<sub>2</sub><sup>-</sup>), and release unreacted substrate. The rearrangements and reactions that follow the transfer of the second electron are very rapid, allowing little or no time for redox partners to dissociate prior to catalysis.

corresponding  $\Delta^{5,16}$ -diene in human beings is not known. Finally, small amounts of other products such as 17 $\alpha$ -hydroxy, C19 steroids are formed by P450c17 in some species, but these activities for the human enzyme are not well-characterized.

### MODELING OF HUMAN CYP17

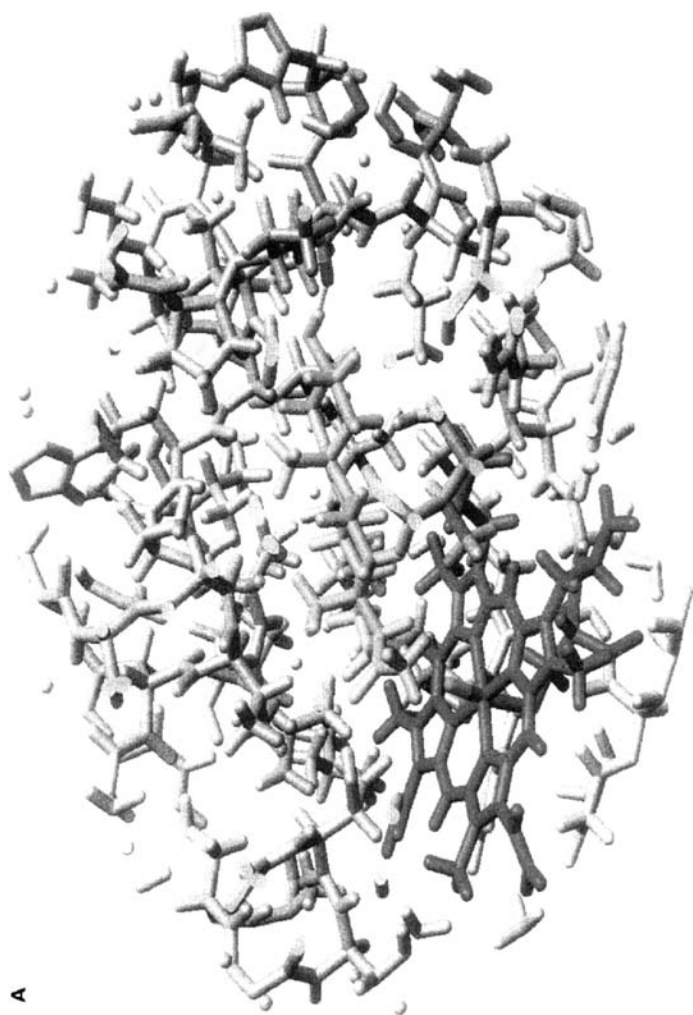
To understand the basis for the remarkable substrate specificity and catalytic versatility of P450c17, a detailed understanding of its tertiary structure is required. Unfortunately all efforts to crystallize P450c17, even when it has been modified and rendered soluble in *E. coli*, have been unsuccessful, as have efforts to crystallize many eukaryotic P450. Thus, substantial work has been directed at building computer graphic models of eukaryotic P450 enzymes based on the crystal structures of several prokaryotic P450s, notably CYP101 (also known as P450cam)(Poulos *et al.*, 1987), CYP108 (also known as P450terp)(Hasemann *et al.*, 1994) and CYP102 (also known as P450BMP)(Ravichandran *et al.*, 1993). This has led to highly successful models of CYP19 (Graham-Lorence *et al.*, 1995) and CYP2B4 (Chang *et al.*, 1997). In the case of P450c17, three groups reported similar models between 1990 and 1997, all of which featured a bilobed substrate binding pocket in which the steroid was

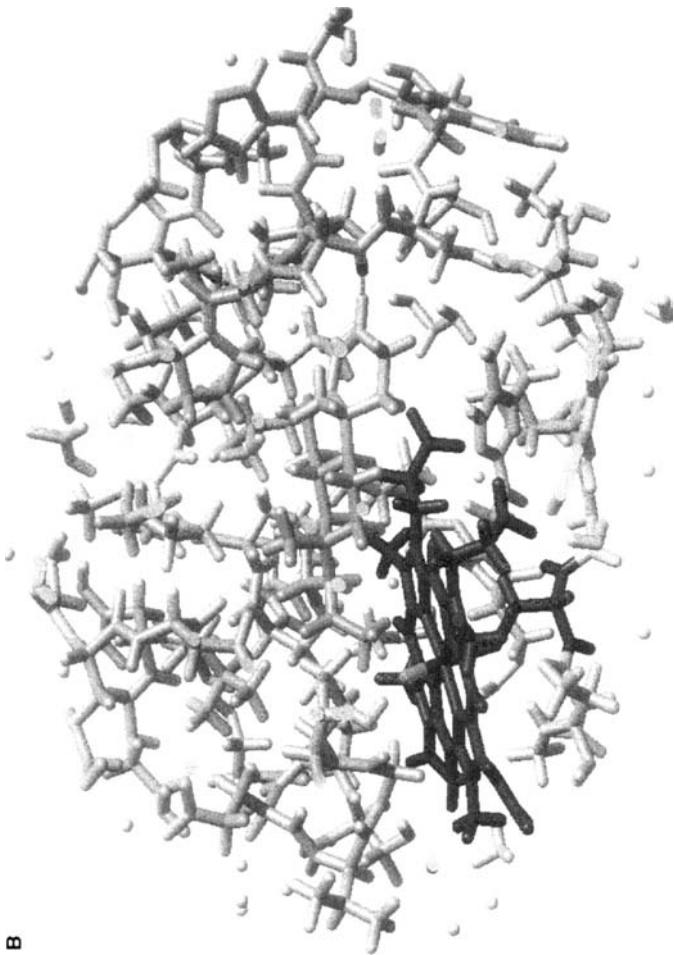
presumed to have the D-ring and C-17 carbon over the heme with A-ring in one lobe of the pocket for the hydroxylase reaction, then moving the A-ring to the other lobe for the 17,20-lyase reaction (Laughton *et al.*, 1990; Lin *et al.*, 1994; Burke *et al.*, 1997). Only one of these models was tested by site-directed mutagenesis, and few of this model's predictions were confirmed experimentally (Lin *et al.*, 1994).

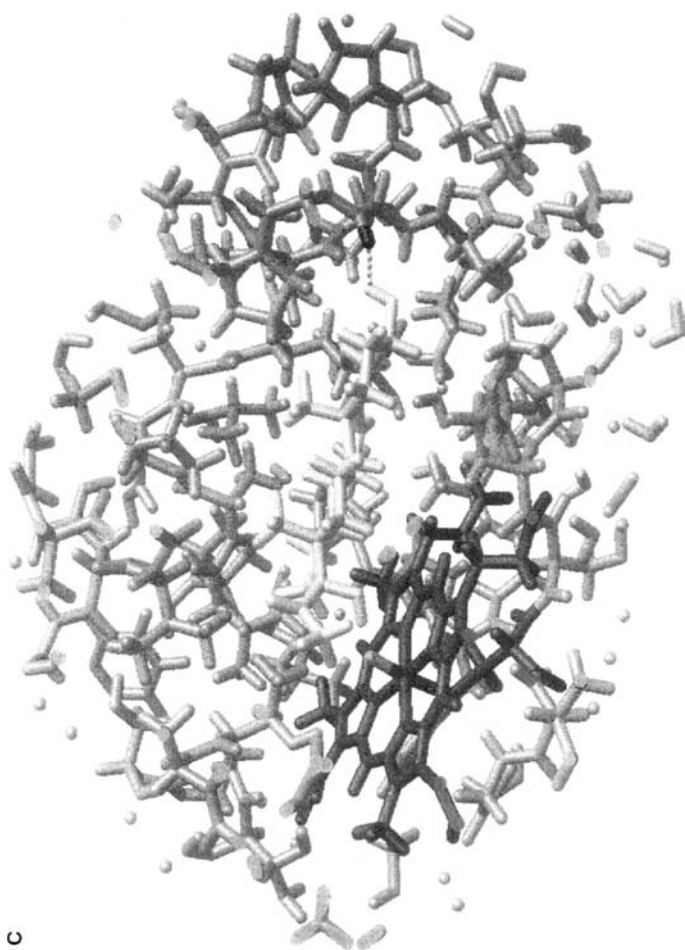
More recent modeling efforts based on the X-ray crystal structure of GYP 102 have proven more robust (Lewis and Lee-Robichaud, 1998; Auchus and Miller, 1999). Our current model uses a combination of the CYP102-template, amino acid alignment procedures based on regions of predicted secondary structure rather than on computationally predicted amino acid sequence similarities, and makes extensive use of molecular dynamics simulations at 300°K using a Cray 3TE super-computer (Auchus, 1998; Auchus and Miller, 1999); the resulting final hydrated model has a total free energy of  $-1.8 \times 10^4$  kcal/mol, which is very similar to the free energy of the GYP 102 crystal structure itself ( $-1.6 \times 10^4$  kcal/mol)(Auchus and Miller, 1999), and is an order of magnitude more stable than the apparently next best model (Lewis and Lee-Robichaud, 1998).

Unlike the previous models, our current model accommodates only a single orientation for all of the steroid substrates and reactions (Figure 9.3). This result suggests that the two principal P450c17 reactions proceed not via grossly different substrate orientations and chemistries, but rather via similar mechanisms; we have proposed that both the hydroxylase and lyase reactions proceed through a ferryl oxene mechanism (Auchus and Miller, 1999). Isotopic labeling studies are consistent with mechanisms involving the nucleophilic attack of a ferryl peroxide on the C-20 carbonyl of the hydroxysteroid, forming a covalent enzyme-steroid intermediate whose decomposition yields the observed products (Akhtar *et al.*, 1994). However, these studies do not exclude mechanisms that involve the same ferryl oxene used in the hydroxylase reactions, in which a steroid hydroxyl radical at C-17 fragments directly without a covalent intermediate (Figure 9.4). Indeed, recent experiments demonstrate that little free  $\text{H}_2\text{O}_2$  is formed during catalysis (Brock and Waterman, 1999) and that  $\text{H}_2\text{O}_2$  itself cannot reconstitute 17,20-lyase activity (Auchus and Miller, 1999), findings that cast doubt on the ferryl peroxide mechanisms. Finally, in addition to being energetically favorable and yielding excellent scores in various model evaluation programs, our current model also accurately predicts the activities of all known natural and synthetic site-directed mutants of P450c17. These mutations are discussed in detail later in this chapter.

Surprisingly, few efforts to develop pharmacological inhibitors of P450c17 have been reported (Stevenson *et al.*, 1991). Nonspecific inhibitors of human P450s, such as aminoglutethimide and ketoconazole, have been used clinically but there are no FDA-approved selective inhibitors of P450c17 as is the case for aromatase (Brodie *et al.*, 1999). It was thought that medroxyprogesterone acetate (MPA) and megestrol acetate (MEG) both inhibit P450c17 because MPA and MEG bind with high affinity to rat testis P450s (Barbieri and Ryan, 1980), but MPA minimally inhibits human P450c17 at concentrations up to 100 $\mu\text{M}$  (Lee *et al.*, 1999), and MEG has no effect on P450c17 activity at 100 $\mu\text{M}$  (unpublished observation). It was reported that P450c17 metabolized dexamethasone (Tomlinson *et al.*, 1997), but this metabolism is + probably performed by a renal P450 enzyme. Using recombinant P450c17, we recently showed that dexamethasone is not a

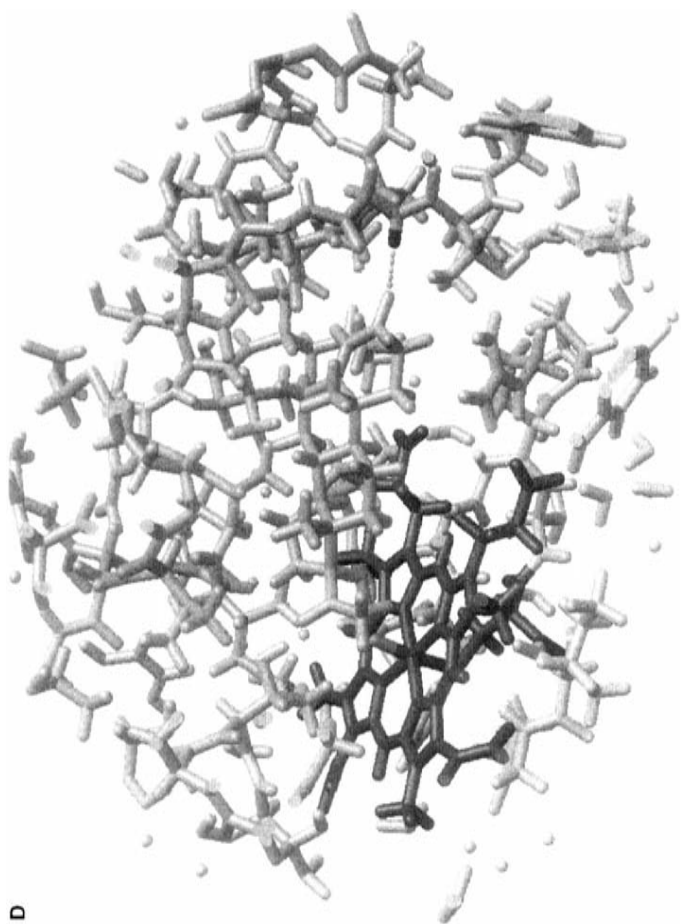




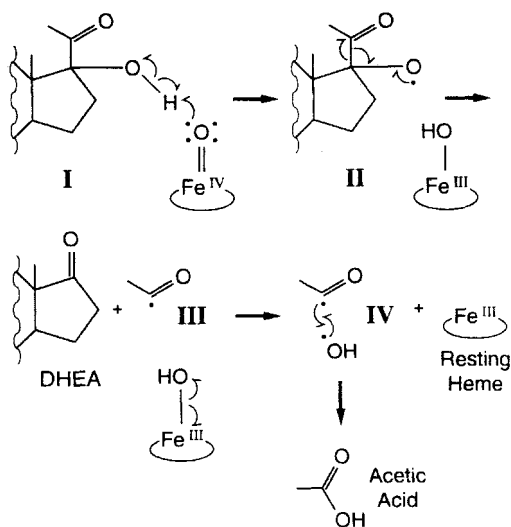


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**Figure 9.3 Positioning of steroid substrates in the active site of p450c17, as predicted by our molecular modeling studies.** The steroid is shown in yellow: Panel A, pregnenolone; panel B, progesterone; panel C, 17 $\alpha$ -hydroxypregnenolone; Panel D 17 $\alpha$ -hydroxypregnenolone. The heme group is shown in red with the ferryl oxene oxygen atom in green, and the protein is in gray. The steroid is positioned through interaction of steroid carbon #3 groups with glycine #95. A hydrogen bond forms from the C-3 hydroxyl hydrogen of the  $\Delta^5$ -steroids pregnenolone or 17-hydroxypregnenolone to the carbonyl oxygen of Gly95 (panels A and C), or from the C-3 carbonyl oxygen of the  $\Delta^4$  steroids progesterone or 17-hydroxypregnenolone to the amide hydrogen of Gly95 (panels B and D), as shown by the dotted lines. Images were generated with the *neon* option of the Midas Plus graphics system from structure pDBID:2C17 ([www.resb.org](http://www.resb.org))(Auchus and Miller, 1999). (See [color plate 1](#))



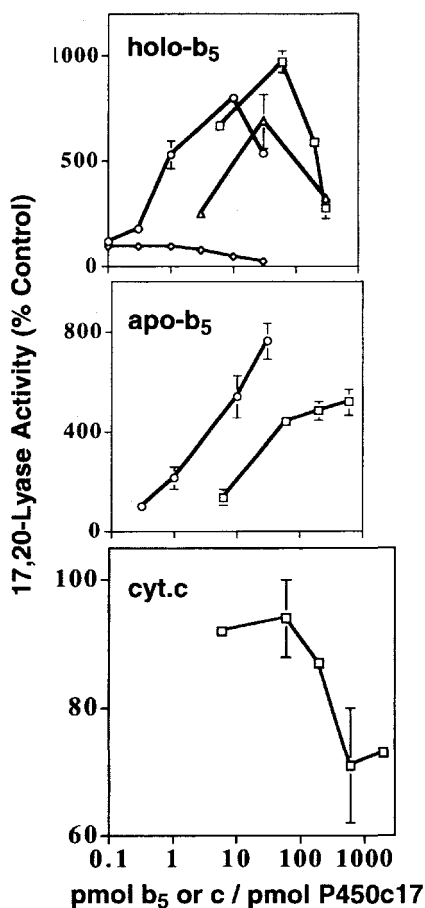
**Figure 9.4 Proposed ferryl oxene mechanism for the 17,20-lyase reaction.** The ferryl oxene abstracts the hydrogen from the hydroxyl on carbon #17 of 17-hydroxypregnenolone (I), generating a C-17 hydroxyl radical ( $\text{O}^*$ ) and a heme (ferric) hydroxyl (II). This unstable radical spontaneously degrades into DHEA and an acetyl radical ( $\text{H}_3\text{C}-\text{C}=\text{O}$ ) (III). Reaction with the heme hydroxyl yields acetic acid, returning the heme to its resting state, where it then binds a water molecule (not shown).

substrate and is only a very weak competitive inhibitor of P450c17 ( $K_i=87\mu\text{M}$ ), a property which cannot account for any known therapeutic effect of dexamethasone (Lee *et al.*, 1999).

#### REDOX PARTNERS

The role of electron transfer proteins OR and cytochrome  $b_5$  (collectively referred to as “redox partners”) in the regulation of P450c17 activities is of considerable interest. Hall and colleagues showed that increasing the ratio of rat OR to porcine P450c17 in a reconstitution assay using purified, detergent-solubilized proteins preferentially increased the 17,20-lyase activity (Yanagibashi and Hall, 1986). Consistent with this, Hall also found a higher molar ratio of OR to P450c17 in bovine testis, where virtually all steroids undergo both 17 $\alpha$ -hydroxylation and 17,20 bond scission, but there was a lower ratio of OR to P450c17 in bovine adrenal, which primarily produces 17-hydroxy, C21 steroids (Yanagibashi and Hall, 1986). Similarly, increasing the ratio of OR to human P450c17 in transfected COS-1 cells increases the ratio of 17,20-lyase to 17 $\alpha$ -hydroxylase activity (Lin *et al.*, 1993).

Cytochrome  $b_5$  has been proposed as an alternate to OR for electron transfer to P450c17, particularly for the 17,20-lyase reaction. Addition of purified rabbit liver  $b_5$  to porcine P450c17 with rat OR increased the 17, 20-lyase activity (Auchus and Miller, 1999), and both rat (Katagiri *et al.*, 1995) and pig (Lee-Robichaud *et al.*, 1995) liver  $b_5$  augment the 17,20-lyase activity of recombinant, modified human P450c17. We showed that the stimulatory effect of  $b_5$  on the 17,20-lyase activity of wild-type P450c17 was maintained



**Figure 9.5 Cytochrome  $b_5$  exerts an allosteric effect to stimulate the 17,20-lyase activity of human P450c17.** Activity was examined in microsomes prepared from human adrenal tissue (triangles) or from yeast expressing human P450c17 and co-expressing high (squares), low (circles) or very low (diamond) amounts of human OR. The high, low, and very low OR contents are 223, 33, and 12nmol of cytochrome c reduced  $\times \text{min}^{-1} \times \text{mg}$  of protein $^{-1}$ , respectively. Top: stimulation with holo- $b_5$  maximizes lyase activity at a  $b_5$ : P450c17 molar ratio of 30:1, after which further increases in  $b_5$  inhibit the reaction. However, when OR content is low the inhibitory phase begins at lower  $b_5$ : P450c17 ratios, and when OR is nearly absent, only the inhibitory action of  $b_5$  is observed. Middle: by contrast, apo- $b_5$  stimulates 17,20-lyase activity at all concentrations, and shows no inhibitory phase, whereas co-incubation with cytochrome c produces only an inhibitory phase (Bottom). Thus, the action of  $b_5$  to stimulate 17,20-lyase activity is unrelated to its ability to transfer electrons, and in fact the electron-transfer capacity of small cytochromes inhibits lyase activity (data from Auchus *et al.*, 1998a).

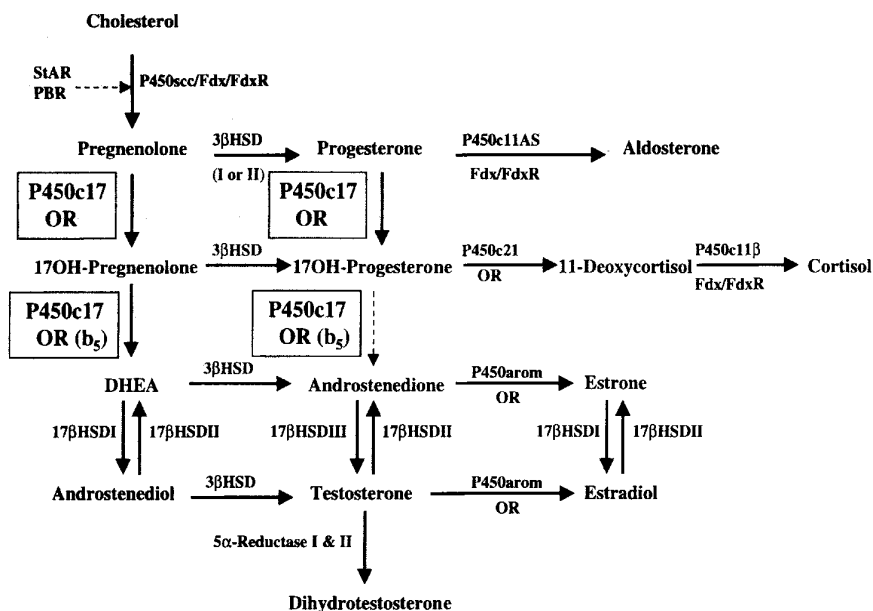
when the human forms of P450c17 and OR were coexpressed in yeast microsomes with and without human  $b_5$  (Auchus *et al.*, 1998a; Geller *et al.*, 1999), demonstrating that the

influence of  $b_5$  is not limited to reconstituted assay systems. The requirement of  $b_5$  for maximal 17,20-lyase activity requires at least a stoichiometric amount of  $b_5$  reductase to P450c17, but very high molar ratios inhibit catalytic activity, probably by competing with P450c17 for electron transfer from OR (Auchus *et al.*, 1998a). In fact, as the abundance of OR is reduced, the inhibitory effect of  $b_5$  occurs at lower molar ratios of  $b_5$  to P450c17 and eventually obscures any stimulatory effect (Figure 9.5). Indeed, apo- $b_5$ , which lacks a heme and therefore cannot participate in electron transfer reactions, stimulates 17,20-lyase activity just as well as holo- $b_5$ , but does not inhibit at higher molar ratios, providing additional evidence that  $b_5$  acts not as an electron donor to P450c17 directly but as an allosteric facilitator of the P450c17•OR catalytic complex (Auchus *et al.*, 1998a). This effect of  $b_5$  on 17,20-lyase activity was not seen in transfected COS-1 cells (Lin *et al.*, 1993), presumably because  $b_5$  is already expressed in these cells at quantities sufficient to stimulate 17,20-lyase activity. In contrast, results in both transfected COS-1 cells (Geller *et al.*, 1999) and yeast microsomes (Auchus *et al.*, 1998a) demonstrate that raising the amount of human OR increased both 17 $\alpha$ -hydroxylase and 17,20-lyase activities, but a differential effect on these two activities has been difficult to demonstrate in these systems.

## PHYSIOLOGY

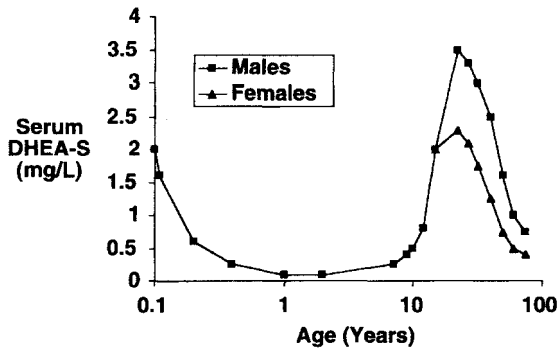
P450c17 is the qualitative regulator of steroidogenesis because its activities determine which class of steroid is made in each cell type (Figure 9.6). In the human adrenal zona glomerulosa, human placenta and rat adrenal, where P450c17 is absent (Voutilainen *et al.*, 1986), only 17-deoxy, C<sub>21</sub> steroids such as progesterone, corticosterone, and mineralocorticoids can be made. These cells reflect the physiology of 17 $\alpha$ -hydroxylase deficiency, with mineralocorticoid-induced hypertension and sexual infantilism. In the human adrenal zona fasciculata, the 17 $\alpha$ -hydroxylase activity of P450c17 enables the biosynthesis of cortisol, the principal human glucocorticoid. However, adrenal 17 $\alpha$ -hydroxylase is not strictly required for glucocorticoid production because corticosterone provides adequate glucocorticoid activity in patients with 17 $\alpha$ -hydroxylase deficiency, just as occurs in rodents, whose adrenal glands do not express P450c17. In the adrenal zona reticularis and in the gonads, the high ratio of 17,20-lyase to 17 $\alpha$ -hydroxylase activities results in the synthesis of C<sub>19</sub> precursors of sex steroids. Thus, the types of steroids made by a given steroidogenic cell or tissue are determined by the activities of P450c17 present; by contrast, the total quantity of steroids is mainly modulated by StAR (acutely) and P450scc (chronically)(for reviews see Miller, 1988; Stocco and Clark, 1996; Miller, 1998).

As it is clear that a single enzyme, P450c17, catalyzes both 17 $\alpha$ -hydroxylase and 17,20-lyase activities, the presence of only (or predominantly) the hydroxylase activity presents a biochemical conundrum: how does the cell stop the reaction after the hydroxylase activity? This conundrum is further accentuated when one considers the physiological evidence that the ratio of lyase to hydroxylase activity not only differs among cell types, but can be developmentally regulated within a single cell type. That cell type is the adrenal zona fasciculata/reticularis, and the regulation is evidenced in the phenomena of adrenarche and adrenopause (Figure 9.7).



**Figure 9.6 Major pathways of human steroidogenesis.** Cholesterol is converted to pregnenolone by P450<sub>scc</sub> (CYP11A) and facilitated by the StAR protein, serving as the quantitative regulator of steroidogenesis (Miller, 1995, 1988; Stocco and Clark, 1996), while P450c17 serves as the qualitative regulator. Top Line: in the absence of P450c17, steroidogenesis is limited to progesterone (e.g., in human placenta), corticosterone (e.g., in rat adrenal zona fasciculata), and aldosterone (adrenal zona glomerulosa). Middle line: when the 17 $\alpha$ -hydroxylase activity, but not the 17,20-lyase activity, of P450c17 is present in the human adrenal zona fasciculata cortisol is produced. Bottom line: when both the 17 $\alpha$ -hydroxylase and 17,20-lyase activities of P450c17 are present 19-carbon precursors of sex steroids are produced. Human P450c17 catalyzes the 17-hydroxylation of the  $\Delta^5$  steroid pregnenolone and of the  $\Delta^4$ -steroid progesterone equally well, but the 17,20-lyase activity exhibits a 100:1 preference for 17-hydroxypregnenolone over 17-hydroxyprogesterone (Auchus *et al.*, 1998a). Thus, essentially all human sex steroids are made from DHEA.

Adrenarche is the rapid and profound rise in the adrenal secretion of DHEA and DHEAS that begins at about age 8 and reaches maximal levels at age 25–30 when the secretion of these C<sub>19</sub> steroids exceeds that of cortisol; thereafter, their secretion slowly decreases (adrenopause), reaching early childhood levels in persons over 70 (Orentreich *et al.*, 1984). Meanwhile, the secretion of cortisol and ACTH (adjusted for body surface area) changes little with age. Adrenarche is a phenomenon unique to human beings, chimpanzees, and possibly some other, large old world monkeys (Cutler *et al.*, 1978; Castracane *et al.*, 1981; Smail *et al.*, 1982); hence no convenient animal model of adrenarche is available. Adrenarche is independent of puberty, gonads, and gonadotropins (Sklar *et al.*, 1980), and its timing appears to correlate roughly with the expansion of the adrenal zona reticularis (Cell *et al.*, 1998). However, the cellular and biochemical events that define adrenarche have



**Figure 9.7 Human serum concentrations of DHEA-sulfate (DHEAS) as a function of age.** The fetal adrenal makes large quantities of DHEAS, which are evident at birth. As the fetal zone of the adrenal involutes, DHEAS concentrations plummet in infancy and remain low until the onset of adrenarche at about age 8. DHEAS concentrations reach maximal values around age 25 then decline slowly with advancing age, returning to childhood levels in the elderly (adrenopause). Note that the abscissa is shown on a logarithmic scale.

remained enigmatic, mainly due to the lack of suitable animal and cellular models (for review see Miller, 1999).

Recent progress in the regulation of 17,20-lyase activity and in the biochemistry of the zona reticularis have begun to elucidate possible mechanisms responsible for adrenarche. In addition to the role of redox partners in regulating 17,20-lyase activity (Lin *et al.*, 1993; Auchus *et al.*, 1998a, b; Miller *et al.*, 1998), serine/ threonine phosphorylation of P450c17 preferentially increases 17,20-lyase activity (Zhang *et al.*, 1995). Thus, a developmentally-programmed rise in P450c17 phosphorylation could contribute to adrenarche. In addition, the expression of  $b_5$  appears to be more abundant in the zona reticularis (Yanase *et al.*, 1998), although its abundance relative to P450c17 itself is not known. A 30:1 molar ratio of  $b_5$  to P450c17 maximizes 17,20-lyase activity (Auchus *et al.*, 1998a; Geller *et al.*, 1999). If the preadrenarchal adrenal gland has low molar ratios of  $b_5$  to P450c17, and the adrenarchal reticularis achieves high ratios, then  $b_5$  abundance could be a defining feature of adrenarche. Region-specific expression of  $b_5$ , combined with low abundance of 3 $\beta$ -hydroxysteroid dehydrogenase type II (3 $\beta$ HSDII) in the zona reticularis (Sasano *et al.*, 1990; Endoh *et al.*, 1996; Cell *et al.*, 1998), would drive steroids down the  $\Delta^5$  pathway to DHEA.

Substantially less work has been directed towards the basis of adrenopause. It is likely that at least some of the factors that drive adrenarche reverse themselves during this process but there are no direct experimental data on this point. Furthermore, the physiologic role of adrenarche, adrenopause, and DHEA are still debated, but accumulating evidence suggests that DHEA and DHEAS are important for developing neuronal connections in the fetal brain (Compagnone and Mellon, 1998) and numerous studies indicate that DHEA supplementation in the elderly can have salutary effects on immune function, memory and sense of well-being (Flood *et al.*, 1992; Morales *et al.*, 1994). Nearly 80% of testosterone in women derives from peripheral conversion of DHEA(S) (Arlt *et al.*, 1998), suggesting that adrenal DHEA is an important precursor of anabolic steroids in normal women.

## GENETICS

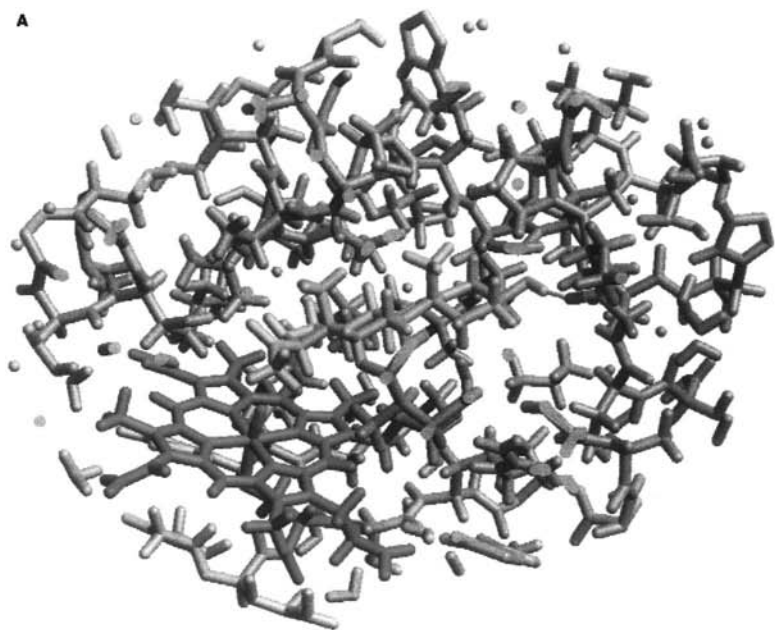
Human P450c17 is encoded by a single gene, formally termed CYP17, that is located on chromosome 10q24.3 (Matteson *et al.*, 1986; Sparkes *et al.*, 1991; Fan *et al.*, 1992) and mRNA that is expressed in the adrenals and gonads (testicular Leydig and ovarian theca cells) (Chung *et al.*, 1987) but (unlike the rodent) not in the placenta or ovarian granulosa cells (Voutilainen *et al.*, 1986). The 6.5kb gene has been sequenced in its entirety, showing it is divided into eight exons that have an organization very similar to the gene for CYP21 (steroid 21-hydroxylase). Amino acid sequence alignments and computational predictions of secondary structure also suggest that the P450c17 and CYP21 proteins have very similar folding and 3-dimensional conformation, despite sharing only 28.9% amino acid sequence identity (Chung *et al.*, 1987; Picado-Leonard and Miller, 1987, 1988; Lin *et al.*, 1994; Auchus and Miller, 1999). In addition to the detailed genetic knowledge of the gene for P450c17, advanced computer graphic modeling of the P450c17 protein (Auchus and Miller, 1999) based on the X-ray crystal structure of P450-BMP (Ravichandran *et al.*, 1993) now permits one to analyze the disease-causing mutations of P450c17 at genetic, protein-structural, and enzymologic levels.

#### DELETIONS, PREMATURE TRUNCATIONS, FRAMESHIFTS AND SPLICING ERRORS

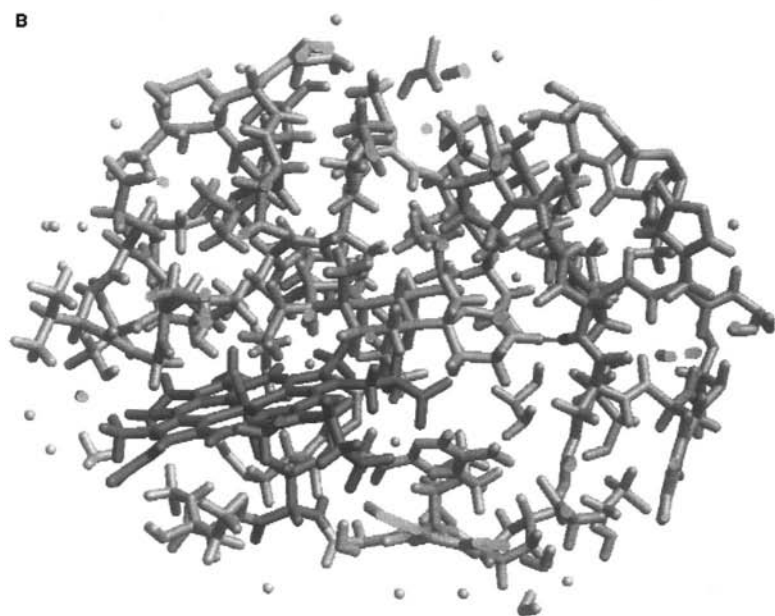
Several deletions encompassing part, but not all of the P450c17 gene have been described (Figure 9.8). The largest deletion reported involves the substitution of 518bp (comprising most of exon 2 and part of exon 3) with 469bp of unknown DNA, thus disrupting the protein near its very beginning and causing complete 17 $\alpha$ -hydroxylase deficiency (Biason *et al.*, 1978). The first P450c17 mutation reported, and possibly the most common, is the duplication of the sequence CATC following Ile479 (Kagimoto *et al.*, 1988). This 4bp duplication, originally observed in Canadian Menonites (Kagimoto *et al.*, 1989) and subsequently found in at least six Dutch Frieslander families (Imai *et al.*, 1992), leaves 95% of the protein unaffected (including the heme-binding region) and creates a mutant P450c17 that has an altered sequence in only its last 25 residues and is truncated three residues prematurely, yet is wholly devoid of enzymatic activity. The crucial nature of the carboxy terminus of P450c17 is also shown by the complete absence of activity in the 9bp in-frame deletion of residues Asp487, Ser488, and Phe489 (Fardella *et al.*, 1993). Because the amino acid sequences of the extreme carboxy end of P450c17 and CYP21 are wholly different, these genetic and enzymologic studies did not tell why this region of the protein is so important or what function it serves.

However, our model of human P450c17 suggests why the enzyme is so sensitive to alterations in its carboxy-terminus (Auchus and Miller, 1999). After the most carboxy-terminal helix (the L-helix) found in all the x-ray crystal structures of P450 enzymes (Hasemann *et al.*, 1995), the polypeptide chain completes the final two pairs of strands of two  $\beta$ -sheets before returning to the protein surface and terminating. In forming these P-sheet strands, these last residues fold down from the protein surface into the protein core to a point above the heme, forming the "top" of the substrate-binding pocket before turning back on itself and exiting the protein core (Auchus and Miller, 1999). Thus, the last 48 residues of

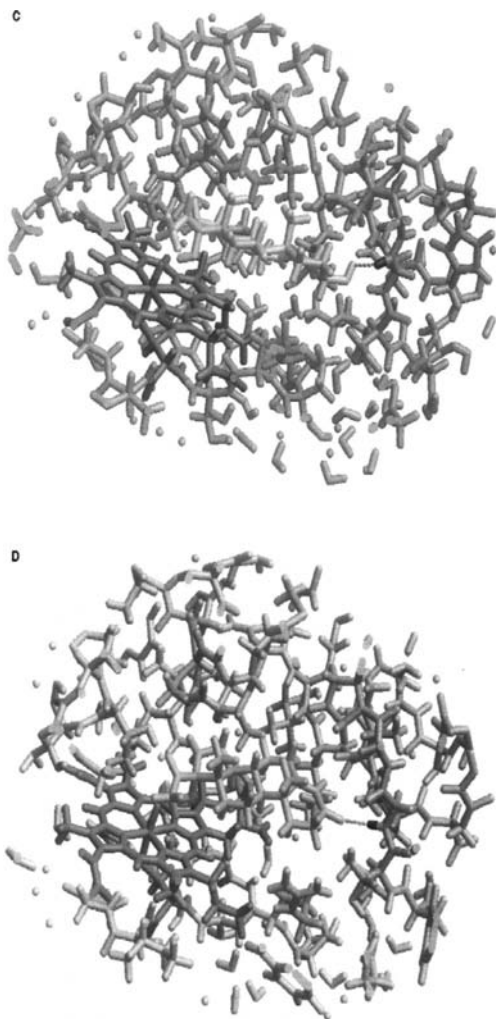
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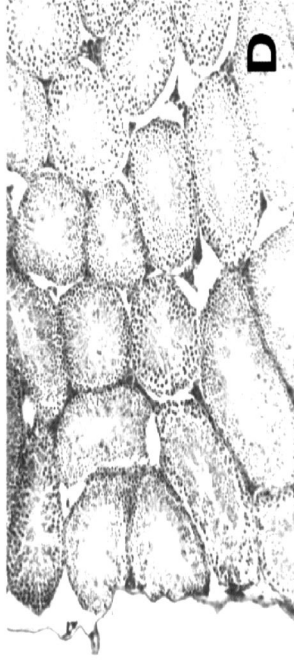
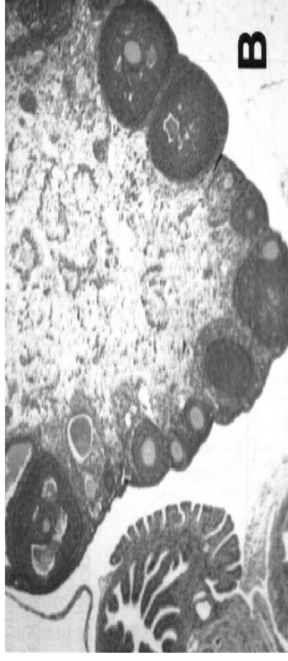
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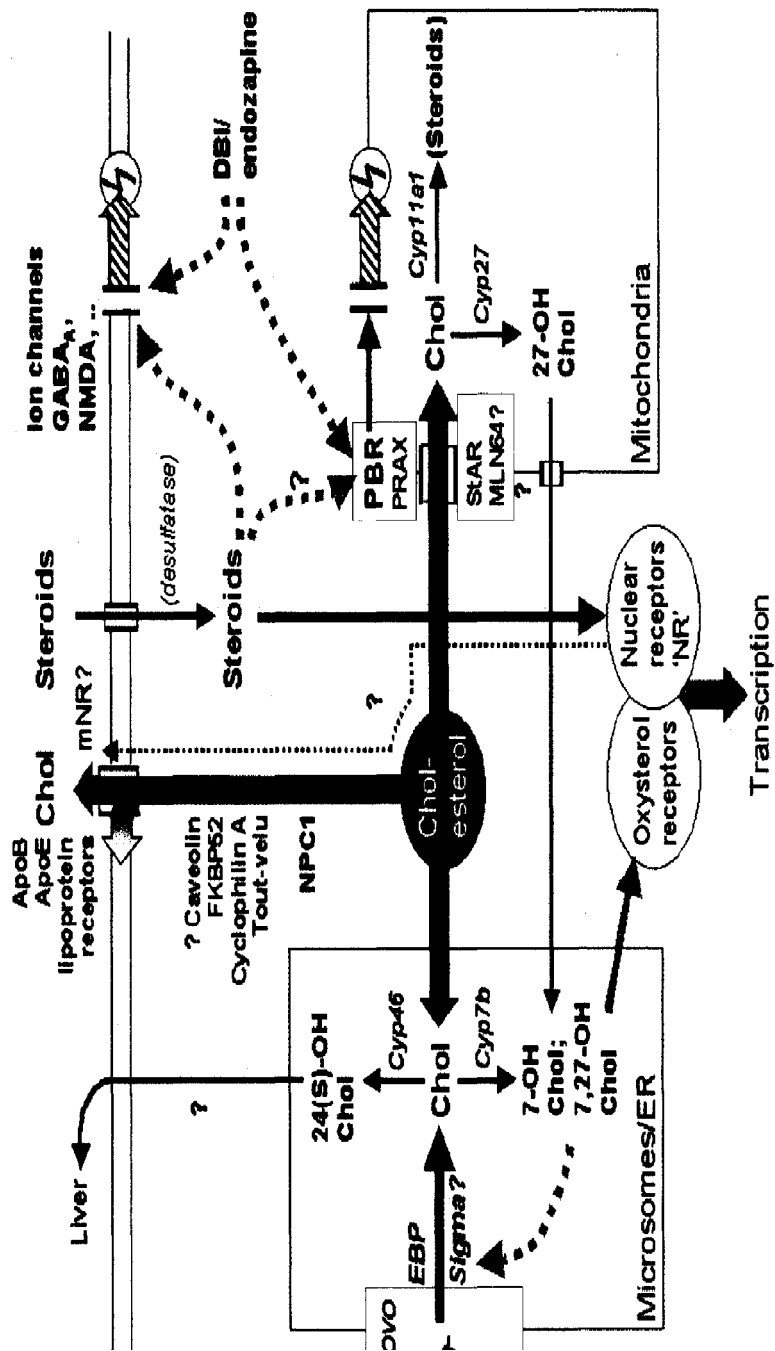




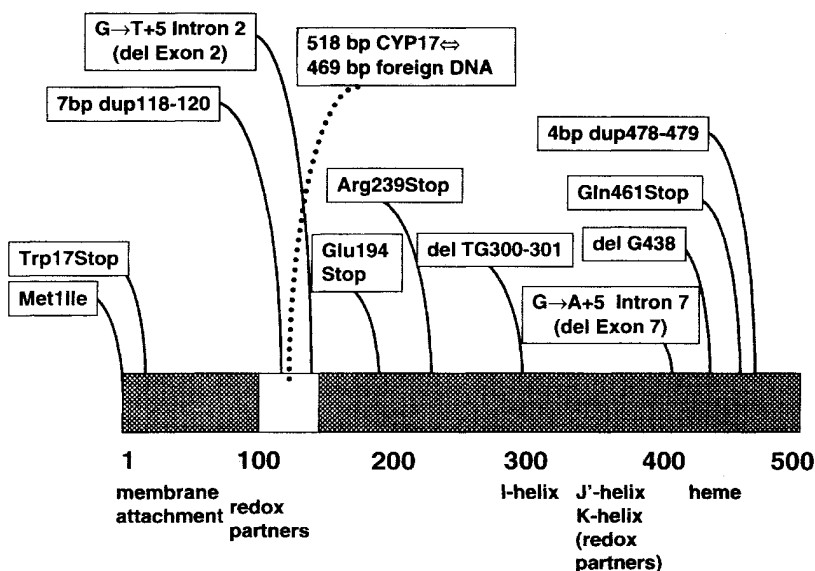
**Color plate 1 Positioning of steroid substrates in the active site of P450c17, as predicted by our molecular modeling studies.** The steroid is shown in yellow: Panel A, pregnenolone; Panel B, progesterone; Panel C; 17 $\alpha$ -hydroxypregnenolone; Panel D 17 $\alpha$ -hydroxyprogesterone. The heme group is shown in red with the ferryl oxene oxygen atom in green, and the protein is in gray. The steroid is positioned through interactions of steroid carbon #3 groups with glycine #95. A hydrogen bond forms from the C-3 hydroxyl hydrogen of the  $\Delta^5$ -steroids pregnenolone or 17-hydroxypregnenolone to the carbonyl oxygen of Gly95 (panels A and C), or from the C-3 carbonyl oxygen of the  $\Delta^4$  steroids progesterone or 17-hydroxyprogesterone to the amide hydrogen of Gly95 (panels B and D), as shown by the dotted lines. Images were generated with the *neon* option of the Midas Plus graphics system from structure pDBID:2C17 ([www.resb.org](http://www.resb.org)) (Auchus and Miller, 1999). (See pages 265–268)



**Color plate 2** Optical micrographs of ovaries (A and B) and testes (C and D) of wild-type (A and C) and ArKO (B and D) mice at 12–14 weeks of age. Magnification: A,  $\times 10$ ; B–D,  $\times 20$ . (See page 293)



**Color plate 3** Pathways of sterol and steroid signalling in brain. Chol, cholesterol; DBI, diazepam binding inhibitor; EBP, emopamil binding protein; ER, endoplasmic reticulum; PBR, Peripheral benzodiazepine receptor; (Z), membrane depolarization. (See page 434)



**Figure 9.8** Deletions and insertions causing major disruptions of P450c17. The bar indicates the normal 508 amino acid protein with certain functionally important regions indicated. The terms “del” and “dup” refer to nucleotide deletions and duplications, with the corresponding nucleotide numbers from the cDNA sequence. The open box designates a  $\sim 0.5$  kb DNA substitution mutation (Biaison *et al.*, 1991). All these mutations cause a complete absence of all 17 $\alpha$ -hydroxylase/17,20-lyase enzymatic activity.

P450c17 are involved in an extended  $\beta$ -sheet structure, the entirety of which forms a scaffold for a region of the protein critical for proper substrate binding and subsequent catalysis. Thus, the Friesian mutation altering the reading frame after Ile479 (Kagimoto *et al.*, 1988), the Thai mutation deleting residues 487–489 (Fardella *et al.*, 1993) and the mutant Gln461 $\rightarrow$ stop (Yanase *et al.*, 1992), which all retain the heme-binding site, disrupt or lack this critical stretch of residues required for activity.

Mutation delTG300, 301 changes the reading frame and alters the codon usage beginning within exon 5 (Monno *et al.*, 1997), and mutation 7bp dup 120 interferes with the reading frame from exon 2 onward (Yanase *et al.*, 1990). The mutation Trp 17 $\rightarrow$ stop was found in a homozygous patient (Yanase *et al.*, 1988) and in a heterozygous patient (Suzuki *et al.*, 1998). Both mutations, Glu194 $\rightarrow$ stop and Arg239 $\rightarrow$ stop, represent separate alleles in a single patient with complete 17 $\alpha$ -hydroxylase deficiency (Rumsby *et al.*, 1993). These three early truncations delete the heme-binding region as well as residues important for substrate and redox partner binding; hence, these mutations are not informative for structure/function studies.

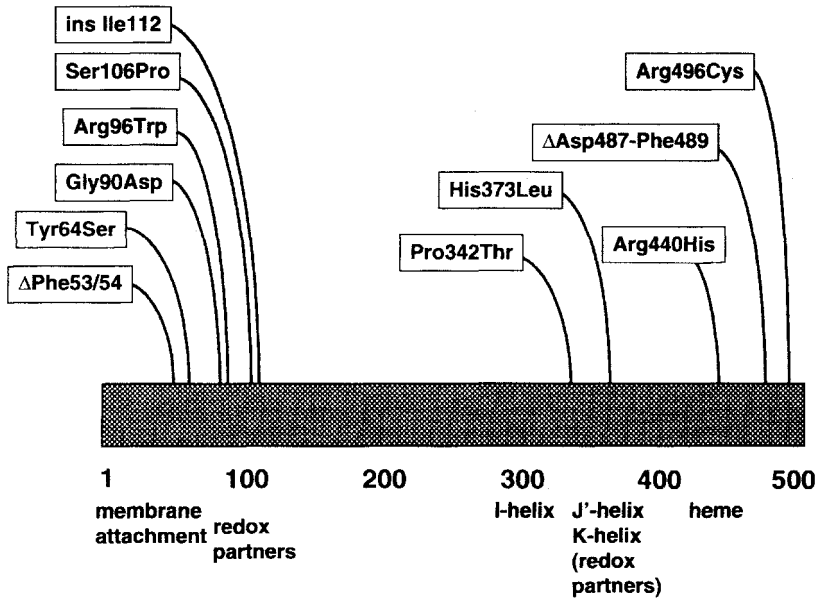
In the compound heterozygote bearing the Trp 17 $\rightarrow$ stop mutation, the other allele was a G to T substitution at nucleotide +5 in intron 2 (Suzuki *et al.*, 1998). This mutation disrupted a splice donor site, and exon 2 of this allele is deleted during RNA processing

(“exon skipping”), which the authors demonstrated by RT-PCR of testicular RNA (Suzuki *et al.*, 1998). The loss of exon 2 causes a frameshift after amino acid 99 with premature termination at codon 175, and complete loss of activity. An analogous G to A substitution at position +5 of intron 7 in a homozygous patient results in the excision of exon 7 during RNA processing, as proven by minigene expression studies in transfected COS-1 cells (Yamaguchi *et al.*, 1997). The excision of exon 7 alters the reading frame of the crucial exon 8, which changes residues 381 onward and causes premature termination at codon 410, upstream from the heme-binding region that is required for activity. Another homozygous patient was found to harbor a del G mutation within codon 438 (Oshiro *et al.*, 1995). The mutant gene encodes a protein with the Gly-Pro-Arg-Ser-Cys-Ile motif at residues 438–443 in the heme binding region converted to Asp-Leu-Ala-Pro-Val-Stop, which destroys all enzymatic activity. Lastly, an extreme example of premature termination occurs in the ATG→ATC substitution that eliminates the initiating methionine codon at position 1 of a patient with complete 17 $\alpha$ -hydroxylase deficiency (Satoh *et al.*, 1998).

#### AMINO ACID SUBSTITUTIONS—COMBINED 17 $\alpha$ - HYDROXYLASE/17,20-LYASE DEFICIENCY

Amino acid substitution mutations are “site-directed mutagenesis experiments of nature”, and provide some of the clearest insights into the structure/function relationships of P450c17 (Figure 9.9). Two reported substitution mutations appear to involve defective binding of heme. The first reported mutation in P450c17 that impairs heme binding was His373Leu. When expressed in *E. coli* this protein lacks the classical P450 difference spectrum, providing excellent evidence that the protein does not bind heme (Monno *et al.*, 1993). However, computer modeling studies predict that His373 does not lie near the heme itself (Auchus and Miller, 1999). It is quite possible that mutation His373Leu introduces a substantial structural change elsewhere in the protein that subsequently abolishes heme binding, a hypothesis that is consistent with available information. Mutation Arg440His lies in the heme-binding region two residues away from Cys442, which donates the axial sulfhydryl group to the heme iron (Fardella *et al.*, 1994). By analogy with structures of bacterial P450 enzymes, the positively charged guanidinium group on this Arg is predicted to neutralize the negative charge on one of the two propionic acid groups of the heme moiety. Consequently, substitutions for this arginine are expected to impair heme binding. The analogous mutation of Arg435 in the aromatase enzyme (CYP19) causes severe aromatase deficiency as well (Conte *et al.*, 1994).

The homozygous mutation Ser106Pro has been found in two apparently unrelated Guamanian patients (Lin *et al.*, 1991). This mutation introduces a helix-breaking proline into what is predicted to be the B'-helix, 6 residues removed from Ile112, which forms a lateral boundary of the substrate-binding pocket (Auchus and Miller, 1999). Hence, P450c17 is quite sensitive to perturbations in this region. In fact, the more conservative replacement of Ser106 with Thr, the corresponding residue found in rainbow trout P450c17 (Sakai *et al.*, 1992), also abolishes most enzymatic activity (Lin *et al.*, 1993). Slightly further to the amino terminus are Gly90 and Arg96, which are altered in mutants Gly90Asp and Arg96Trp (LaFlamme *et al.*, 1996). These residues appear to flank strand 2 of  $\beta$ -sheet 1, and Gly95,



**Figure 9.9 Mis-sense mutations and in-frame mutations of P450c17.** Amino acid deletions are designated  $\Delta$ , and insertions are designated ins, followed by the three letter amino acid designation and number. Other conventions as in Figure 9.8.

found in this  $\beta$ -strand, appears to participate directly in substrate binding. During molecular dynamics simulations of  $\Delta^5$  and  $\Delta^4$  substrates bound to our model of P450c17, the 3 $\beta$ -hydroxyl and 3-keto groups of these substrates respectively form hydrogen bonds to the carbonyl group (3 $\beta$ -hydroxy) or the amide hydrogen (3-keto) of Gly95 (Figure 9.3) (Auchus and Miller, 1999). Therefore, it is likely that the 3 mutants Ser106Pro, Arg96Trp, and Gly90Asp all primarily impair substrate binding.

Two additional mutations cause partial loss of both 17 $\alpha$ -hydroxylase and 17,20-lyase activities. A 46XY patient has been described who is a compound heterozygote for mutations Tyr64Ser and insIle112 (Imai *et al.*, 1993). Whereas the insIle112 mutation is devoid of measurable activity, the Tyr64Ser mutation retains about 15% of wild-type 17 $\alpha$ -hydroxylase and 17,20-lyase activities (Imai *et al.*, 1993). A second, less severe mutation, Pro342Thr, has been described in a 46XY patient with the premature truncation Arg 239 $\rightarrow$ stop at the other allele. This patient had ambiguous genitalia despite about 20% residual activity at this allele (Ahlgren *et al.*, 1992).

The structural consequences of mutations Tyr64Ser and Pro342Thr are more subtle than the more severe perturbations described for the mutations causing complete 17 $\alpha$ -hydroxylase deficiency. Tyrosine 64 lies in  $\beta$ -sheet 1, which is involved in substrate binding and membrane attachment. Because serine is considerably less hydrophobic than tyrosine, this substitution might interfere with attachment and/or hydrophobic packing interactions. Proline 342 is predicted to start the J' helix, a section of the protein known to participate in

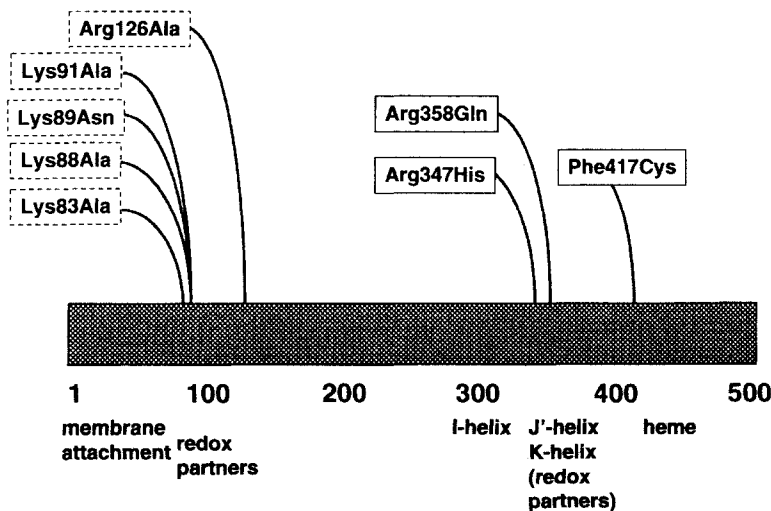
redox partner interactions (Ravichandran *et al.*, 1993). Because proline residues can adopt a range of geometries unique among the amino acids, any substitution involving proline can be catastrophic. In the case of Pro342Thr, however, it appears that the substituted threonine can accommodate a reasonably similar geometry to compensate for the loss of proline.

Two other in-frame mutations that wholly ablate 17 $\alpha$ -hydroxylase activity merely add or delete a single residue. The mutant ins11el 12, in which the ATC codon is repeated in-frame, is completely inactive (Imai *et al.*, 1993). Although Ile112 is quite distant from the C-terminal  $\beta$ -sheet region in linear sequence, Ile112 is predicted to form a different portion (a lateral edge) of the substrate-binding pocket. Consequently, the insertion of an extra residue at this site disrupts the dimensions of the substrate-binding pocket. By contrast, the mutant  $\Delta$ Phe53/54 excises one of the two adjacent phenylalanine residues at positions 53 and 54 (Yanase *et al.*, 1989). These residues appear to line the hydrophobic cleft between the P-sheet and the  $\alpha$ -helical domains of P450c17 and may comprise part of the substrate access channel; the absence of one of these Phe residues eliminates most but not all 17 $\alpha$ -hydroxylase and 17,20-lyase activities (Yanase *et al.*, 1991). Consequently, this  $\Delta$ Phe53/54 mutation is associated with a milder clinical phenotype. This mutation has been described in four apparently unrelated Japanese patients (Miura *et al.*, 1996), suggesting it is due to a genetic founder effect.

#### MUTATIONS CAUSING ISOLATED 17,20-LYASE DEFICIENCY

A heterozygous Swiss patient illustrates several key concepts about P450c17 deficiencies. One of the patient's two alleles contained the Gln461 $\rightarrow$ stop mutation, and the other contained the substitution Arg496Cys. Clinically, the patient appeared to have "isolated 17, 20-lyase deficiency" as a child (Zachmann *et al.*, 1982), but when restudied as an adult, the diagnosis of "partial, combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency" appeared more appropriate (Zachmann *et al.*, 1992). When the mutant proteins were studied in transfected cells, the Gln461 $\rightarrow$ stop mutant was inactive as predicted, while the Arg496Cys mutant retained <10% of both 17 $\alpha$ -hydroxylase and 17,20-lyase activities (Yanase *et al.*, 1992). It is again remarkable that in an amino acid substitution only 13 residues from the carboxy-terminus eliminates over 90% of enzymatic activity, but Arg496 appears to be the last residue in the tandem- $\beta$ -sheet tongue that forms the roof of the substrate-binding pocket (Auchus and Miller, 1999). Thus the Arg to Cys substitution at this distal location results in a substantial but incomplete loss of activity. This patient was the first patient with presumed isolated 17,20-lyase deficiency to be studied at a molecular genetic level, which showed that the patient could not have an isolated deficiency of 17,20-lyase activity. These sobering results raised the question of whether isolated 17,20-lyase deficiency truly existed.

Dr. Berenice Medonça studied the steroid secretion of two 46XY patients from rural Bahia, Brazil who showed clinical and laboratory evidence of isolated 17,20-lyase deficiency. These patients, from two unrelated but consanguineous families, had genital ambiguity, normal 17OHCS excretion rates, but markedly reduced C<sub>19</sub> steroid production. Each patient was homozygous for an amino acid substitution, either Arg347His or Arg358Gln (Figure 9.10) (Geller *et al.*, 1997). When studied in transfected cells, these mutants retained the capacity to hydroxylate pregnenolone and progesterone, but neither mutant could



**Figure 9.10** Amino acid substitutions causing isolated 17,20-lyase deficiency. The mutations at Arg347, Arg358 and Phe417 shown in solid boxes were identified in patients; the other mutations shown in dotted boxes were created *in vitro*. Conventions as in Figures 9.8 and 9.9.

convert 17-hydroxypregnenolone to DHEA unless an excess of both OR and  $b_5$  was supplied by co-transfection (Geller *et al.*, 1999). When studied in yeast microsomes, a trace of 17,20-lyase activity was demonstrated, and the ability of  $b_5$  to augment 17,20-lyase activity was reduced. Competition experiments conclusively demonstrated that 17-hydroxypregnenolone binds to the mutant enzymes with an affinity equivalent to that of the wild-type enzyme, proving the surprising observation that these mutations did not affect substrate access or substrate binding (Geller *et al.*, 1997, 1999). Isolated 17,20-lyase deficiency did exist, but this selective loss of 17,20-lyase activity did not result from mutations in the enzyme's active site.

To understand the biochemical and biophysical basis of these extraordinary mutations in P450c17, we again turned to computational chemistry for insight. Arginines 347 and 358 are located near or at the carboxy-termini of the J' and K helices, in a region of the protein where the presence of highly conserved residues and lack of length discrepancies permit confident structural modeling on the P450BMP template (Auchus and Miller, 1999). Both Arg347 and Arg358 lie beneath the heme ring on the surface of P450c17 that interacts with redox partners; mutations Arg347His and Arg358Gln neutralize some of the positive charges predicted to lie on this surface (Geller *et al.*, 1997). Curiously, the neutralization of the corresponding arginines 346 and 357 of rat P450c17 had been studied previously by site-directed mutagenesis; these mutants (Arg346Ala in particular) also exhibited preferential loss of 17,20-lyase activity (Kitamura *et al.*, 1991), and a similar result was obtained for the Arg347Ala mutation in human P450c17 (Lin *et al.*, 1994). These residues were targeted because they were located in a contiguous series of amino acids (346–369) believed at that time to comprise a consensus steroid-hormone binding site (Picado-Leonard and Miller,



1988). Modifications in this region, therefore, were believed to alter substrate binding and perhaps change the selectivity of P450c17 chemistry.

The computer modeling studies, in contrast, suggested that alterations in positive charges on the enzyme's surface—rather than in a discrete string of residues—caused the isolated loss of 17,20-lyase activity. If this hypothesis is true, then neutralization of other positive charges in the redox-partner binding surface, no matter where they are located in the linear sequence of P450c17, should also preferentially impair 17,20-lyase activity. Accordingly, neutralization of other basic residues on this surface such as lysine 89 with the Lys89Asn mutant increased the hydroxylase/lyase ratio 3-fold (Auchus and Miller, 1999). Analogously, alanine substitution of lysines 83, 88, and 91 and of arginine 126 also increased the hydroxylase/lyase ratio by 2 to 4.5-fold (Lee-Robichaud *et al.*, 1998). Given the wide distribution of these residues in the linear sequence of P450c17 but their congregation in the redox-partner binding site, the evidence implicating this surface as essential to 17,20-lyase activity is compelling.

One other reported P450c17 mutation causing isolated 17,20-lyase deficiency is the substitution Phe417Cys (Biaison-Lauber *et al.*, 1997). However, careful studies of this mutation expressed both in yeast and COS-1 cells show that it has no activity at all, and that the encoded protein lacks a P450 Co difference spectrum and hence has not incorporated heme (Gupta *et al.*, 2001). Thus, to date, only mutations of the arginines at positions 347 and 358 have been shown to cause isolated 17,20-lyase deficiency. Finally, a male pseudohermaphrodite has been described with congenital methemoglobinemia due to a mutation in the gene for cytochrome  $b_5$  (Giordano *et al.*, 1994). It is tempting to speculate that the cause of this patient's undermasculization was due to low (but not absent) 17,20-lyase activity with consequent fetal testosterone deficiency, due not to a P450c17 mutation but rather the loss of  $b_5$ , the cofactor protein that stimulates 17,20-lyase activity. Unfortunately, a thorough endocrinologic evaluation of this patient, including circulating steroid hormone concentrations, has not been published. If true, this case would prove the physiologic importance of  $b_5$  in P450c17 chemistry.

## SUMMARY

The reductionist approach to the P450c17 system has revealed how this key enzyme can have multiple functions that are independently regulated. The study of human disease has shown that the biochemical paradigms that have evolved over the nearly 20 years from Hall's first purification are relevant to human biology. The challenges for P450c17 investigators are now to unravel the structural and mechanistic secrets of the enzyme and its interactions with cofactors OR and  $b_5$ . This knowledge will aid our understanding of enigmatic human physiologic processes such as adrenarche and aging and possibly permit the design of drugs that will selectively increase or decrease P450c17•OR interactions, thus permitting better management of human diseases in which P450c17 plays a major role.

## REFERENCES

- Ahlgren, R., Yanase, T., Simpson, E.R., Winter, J.S.D. and Waterman, M.R. (1992) Compound heterozygous mutations (Arg239→Stop, Pro342→Thr) in the CYP17 (P450-17 $\alpha$ ) gene lead to ambiguous external genitalia in a male patient with partial combined 17 $\alpha$ -hydroxylase/ 17,20-lyase deficiency. *J. Clin. Endocrinol. Metab.* **74**, 667–672.
- Akhtar, M., Corina, D., Miller, S., Shyadehi, A.Z. and Wright, J.N. (1994) Mechanism of the acylcarbon cleavage and related reactions catalyzed by multifunctional P-450s: studies on cytochrome P-450 (17 $\alpha$ ). *Biochemistry* **33**, 4410–4418.
- Arlt, W., Justl, H.-G., Callies, F., Reincke, M., Hubler, D., Oettel, M., Ernst, M., Schulte, H.M. and Allolio, B. (1998) Oral dehydroepiandrosterone for adrenal androgen replacement: pharmacokinetics and peripheral conversion to androgens and estrogens in young healthy females after dexamethasone suppression. *J. Clin. Endocrinol. Metab.* **83**, 1928–1934.
- Auchus, R.J. (1998) The use of computational chemistry in the study of sex steroid biosynthesis. *Endocr. Res.* **24**, 541–547.
- Auchus, R.J., Geller, D.H., Lee, T.C. and Miller, W.L. (1998b) The regulation of human P450c17 activity: relationship to premature adrenarche and the polycystic ovary syndrome. *Trends Endocrinol. Metab.* **9**, 47–50.
- Auchus, R.J., Lee, T.C. and Miller, W.L. (1998a) Cytochrome b<sub>5</sub> augments the 17,20-lyase activity of human P450c17 without direct electron transfer. *J. Biol. Chem.* **273**, 3158–3165.
- Auchus, R.J. and Miller, W.L. (1999) Molecular modeling of human P450c17 (17 $\alpha$ -hydroxylase/ 17,20-lyase): insights into reaction mechanisms and effects of mutations. *Mol. Endocrinol.* **13**, 1169–1182.
- Barbieri, R.L. and Ryan, K.J. (1980) Direct effects of medroxyprogesterone acetate (MPA) and magesrol acetate (MGA) on rat testicular steroidogenesis. *Acta Endocrinol.* **94**, 419–425.
- Baron, J., Taylor, W.E. and Masters, B.S.S. (1972) Immunochemical studies on electron chains involving cytochrome P450: the role of the iron-sulfur protein, adrenodoxin in mixed function oxidation reactions. *Arch. Biochem. Biophys.* **150**, 105–115.
- Biason, A., Mantero, F., Scaroni, C., Simpson, E.R. and Waterman, M.R. (1991) Deletion within CYP17 gene together with insertion of foreign DNA is the cause of combined complete 17 $\alpha$ -hydroxylase/17,20-lyase deficiency in an Italian patient. *Mol. Endocrinol.* **5**, 2037–2045.
- Biason-Laubier, A., Leiberman, E. and Zachmann, M. (1997) A single amino acid substitution in the putative redox partner-binding site of P450c17 as cause of isolated 17,20-lyase deficiency. *J. Clin. Endocrinol. Metab.* **82**, 3807–3812.
- Biglieri, E.G., Herron, M.A. and Brust, N. (1966) 17 $\alpha$ -hydroxylation deficiency in man. *J. Clin. Invest.* **15**, 1945–1954.
- Brock, B.J. and Waterman, M.R. (1999) Biochemical differences between rat and human cytochrome P450c17 support the different steroidogenic needs of these two species. *Biochemistry* **38**, 1598–1606.
- Brodie, A., Lu, Q. and Long, B. (1999) Aromatase and its inhibitors. *J. Steroid Biochem. Molec. Biol.* **69**, 205–210.
- Burke, D.F., Laughton, C.A. and Neidle, S. (1997) Homology modelling of the enzyme P450 17 $\alpha$ -hydroxylase/17,20-lyase—a target for prostate cancer chemotherapy—from the crystal structure of P450BM-3. *Anti-Cancer Drug Design* **12**, 113–123.
- Campo, S., Stivel, M., Nicolau, G., Monteagudo, C. and Rivarola, M. (1979) Testicular function in postpubertal male pseudohermaphroditism. *Clin. Endocrinol.* **11**, 481–490.
- Castracane, V.D., Cutler Jr., G.B. and Loriaux, D.L. (1981) Pubertal endocrinology of the baboon: Adrenarche. *Am. J. Physiol.* **241**, E305–E309.

- Chang, Y.T., Stiffelman, O.B., Vakser, I.A., Loew, G.H., Bridges, A. and Waskell, L. (1997) Construction of a 3D model of cytochrome P450 2B4. *Protein Eng.* **10**, 119–129.
- Chung, B., Picado-Leonard, J., Haniu, M., Bienkowski, M., Hall, P.P., Shiveley, J.E. and Miller, W.L. (1987) Cytochrome P450c17 (steroid 17 $\alpha$ -hydroxylase/17,20-lyase): cloning of human adrenal and testis cDNAs indicates the same gene is expressed in both tissues. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 407–411.
- Compagnone, N.A. and Mellon, S.H. (1998) Dehydroepiandrosterone: a potential signalling molecule for neocortical organization during development. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4678–4683.
- Conte, F.A., Grumbach, M.M., Ito, Y., Fisher, C.R. and Simpson, E.R. (1994) A syndrome of female pseudohermaphroditism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450arom). *J. Clin. Endocrinol. Metab.* **78**, 1287–1292.
- Cooper, D.Y., Levin, S., Narasimhulu, S., Rosenthal, O. and Estabrook, R.W. (1965) Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. *Science* **145**, 400–402.
- Cutler, G.B., Glenn, M., Bush, M., Hodgen, G.D., Graham, C.E. and Loriaux, D.L. (1978) Adrenarche: a survey of rodents, domestic animals and primates. *Endocrinology* **103**, 2112–2118.
- de Peretti, E., Pradon, M. and Forest, M.G. (1984) 17,20-desmolase deficiency in a female newborn, paradoxically virilized *in utero*. *J. Steroid Biochem.* **20**, 455–458.
- Endoh, A., Kristiansen, S.B., Casson, P.R., Buster, J.E. and Hornsby, P.J. (1996) The zona reticularis is the site of biosynthesis of dehydroepiandrosterone and dehydroepiandrosterone sulfate in the adult human adrenal cortex resulting from its low expression of 3 $\beta$ -hydroxy steroid dehydrogenase. *J. Clin. Endocrinol. Metab.* **81**, 3558–3565.
- Fan, Y.S., Sasi, R., Lee, C., Winter, J.S.D., Waterman, M.R. and Lin, C.C. (1992) Localization of the human CYP17 gene (cytochrome P450 17 $\alpha$ ) to 10q24.3 by fluorescence *in situ* hybridization and simultaneous chromosome banding. *Genomics* **14**, 1110–1111.
- Fardella, C.E., Hum, D.W., Homoki, J. and Miller, W.L. (1994) Point mutation Arg440 to His in cytochrome P450c17 causes severe 17 $\alpha$ -hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **79**, 160–164.
- Fardella, C.E., Zhang, L.H., Mahachoklertwattana, P., Lin, D. and Miller, W.L. (1993) Deletion of amino acids Asp487-Ser488-Phe489 in human cytochrome P450c17 causes severe 17 $\alpha$ -hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **77**, 489–493.
- Flood, J.F., Morley, J.E. and Roberts, E. (1992) Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1567–1571.
- Forest, M.G., Lecornu, M. and de Peretti, E. (1980) Familial male pseudohermaphroditism due to 17,20-desmolase deficiency. I. *In vivo* endocrine studies. *J. Clin. Endocrinol. Metab.* **50**, 826–833.
- Gell, J.S., Carr, B.R., Sasano, H., Atkins, B., Margraf, L., Mason, J.I. and Rainey, W.E. (1998) Adrenarche results from development of a 3 $\beta$ -hydroxysteroid dehydrogenase-deficient adrenal reticularis. *J. Clin. Endocrinol. Metab.* **83**, 3695–3701.
- Geller, D.H., Auchus, R.J., Mendonca, B.B. and Miller, W.L. (1997) The genetic and functional basis of isolated 17,20-lyase deficiency. *Nat. Genet.* **17**, 201–205.
- Geller, D.H., Auchus, R.J. and Miller, W.L. (1999) P450c17 mutations R347H and R358Q selectively disrupt 17,20-lyase activity by disrupting interactions with P450 oxidoreductase and cytochrome b<sub>5</sub>. *Mol. Endocrinol.* **13**, 167–175.

- Giordano, S.J., Kaftory, A. and Steggle, A.W. (1994) A splicing mutation in the cytochrome b<sub>5</sub> gene from a patient with congenital methemoglobinemia and pseudohermaphroditism. *Hum. Genet.* **93**, 568–570.
- Goebelsmann, U., Zachmann, M., Davajan, V., Israel, R., Mestman, J.H. and Mishell, D.R. (1976) Male pseudohermaphroditism consistent with 17,20-desmolase deficiency. *Gynecol. Invest.* **7**, 138–156.
- Graham-Lorence, S., Amarneh, B., White, R.E., Peterson, J.A. and Simpson, E.R. (1995) A three-dimensional model of aromatase cytochrome P450. *Prot. Sci.* **4**, 1065–1080.
- Gupta, M.K., Geller, D.H., and Auchus, R.J. (2001) Pitfalls in characterizing P450c17 mutations associated with isolated 17,20-lyase deficiency. *J. Clin. Endocrinol. Metab.* **86**, 4416–4423.
- Hasemann, C.A., Kurumbail, R.G., Boddupalli, S.S., Peterson, J.A. and Deisenhofer, J. (1995) Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure* **2**, 41–62.
- Hasemann, C.A., Ravichandran, K.G., Peterson, J.A. and Deisenhofer, J. (1994) Crystal structure and refinement of cytochrome P450terp at 2.3 Å resolution. *J. Mol. Biol.* **236**, 1169–1185.
- Haugen, D.A. and Coon, M.J. (1976) Properties of electrophoretically homogeneous phenobarbital-inducible and  $\beta$ -naphthoflavone-inducible forms of liver microsomal cytochrome P-450. *J. Biol. Chem.* **251**, 7929–7939.
- Imai, T., Globerman, H., Gertner, J.M., Kagawa, N. and Waterman, M.R. (1993) Expression and purification of functional human 17 $\alpha$ -hydroxylase/17,20-lyase (P450c17) in *Escherichia coli*. *J. Biol. Chem.* **268**, 19681–19689.
- Imai, T., Yanase, T., Waterman, M.R., Simpson, E.R. and Pratt, J.J. (1992) Canadian Mennonites and individuals residing in the Friesland region of the Netherlands share the same molecular basis of 17 $\alpha$ -hydroxylase deficiency. *Hum. Genet.* **89**, 95–96.
- Kagimoto, K., Waterman, M.R., Kagimoto, M., Ferreira, P., Simpson, E.R. and Winter, J.S.D. (1989) Identification of a common molecular basis for combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency in two Mennonite families. *Hum. Genet.* **82**, 285–286.
- Kagimoto, M., Winter, J.S., Kagimoto, K., Simpson, E.R. and Waterman, M.R. (1988) Structural characterization of normal and mutant human steroid 17 $\alpha$ -hydroxylase genes: molecular basis of one example of combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency. *Mol. Endocrinol.* **2**, 564–570.
- Katagiri, M., Kagawa, N. and Waterman, M.R. (1995) The role of cytochrome b<sub>5</sub> in the biosynthesis of androgens by human P450c17. *Arch. Biochem. Biophys.* **317**, 343–347.
- Kaufman, F., Costin, G., Goebelsmann, U., Stanczyk, F.Z. and Zachmann, M. (1983) Male pseudohermaphroditism due to 17,20-desmolase deficiency. *J. Clin. Endocrinol. Metab.* **57**, 32–36.
- Kitamura, M., Buczko, E. and Dufau, M.L. (1991) Dissociation of hydroxylase and lyase activities by site-directed mutagenesis of the rat P450-17 $\alpha$ . *Mol. Endocrinol.* **5**, 1373–1380.
- Kominami, S., Shinzawa, S. and Takemori, S. (1982) Purification and some properties of cytochrome P-450 for steroid 17 $\alpha$ -hydroxylation and C 17, 20 bond cleavage from guinea pig adrenal microsomes. *Biochem. Biophys. Res. Commun.* **109**, 916–921.
- LaFlamme, N., Leblanc, J.-F., Mailloux, J., Faure, N., Labrie, F. and Simard, J. (1996) Mutation R96W in cytochrome P450c17 gene causes combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency in two French Canadian patients. *J. Clin. Endocrinol. Metab.* **81**, 264–268.
- Larrea, F., Lisker, R., Baanuelos, R., Bermaudez, J.A., Herrera, J., Nauanez-Rasilla, V. and Perez-Palacios, G. (1983) Hypergonadotrophic hypogonadism in an XX female subject due to 17, 20 steroid desmolase deficiency. *Acta Endocrinol.* **103**, 400–405.
- Laughton, C.A., Neidle, S., Zvelebil, M.J.J.M. and Sternberg, M.J. (1990) A molecular model for the enzyme cytochrome P450-17 $\alpha$ , a major target for the chemotherapy of prostatic cancer. *Biochem. Biophys. Res. Commun.* **171**, 1160–1167.

- Lee, T.C., Miller, W.L. and Auchus, R.J. (1999) Medroxyprogesterone acetate and dexamethasone are competitive inhibitors of different human steroidogenic enzymes. *J. Clin. Endocrinol. Metab.* **84**, 2104–2110.
- Lee-Robichaud, P., Akhtar, M.E. and Akhtar, M. (1998) Control of androgen biosynthesis in the human through the interaction of Arg347 and Arg358 of CYP17 with cytochrome b<sub>5</sub>. *Biochem. J.* **332**, 293–296.
- Lee-Robichaud, P., Wright, J.N., Akhtar, M.E. and Akhtar, M. (1995) Modulation of the activity of human 17 $\alpha$ -hydroxylase-17,20-lyase (CYP17) by cytochrome b<sub>5</sub>: endocrinological and mechanistic implications. *Biochem. J.* **308**, 901–908.
- Lewis, D.F. and Lee-Robichaud, P. (1998) Molecular modelling of steroidogenic cytochromes P450 from families CYP11, CYP17, CYP19 and CYP21 based on the CYP102 crystal structure. *J. Steroid Biochem. Molec. Biol.* **66**, 217–233.
- Lin, D., Black, S.M., Nagahama, Y. and Miller, W.L. (1993) Steroid 17 $\alpha$ -hydroxylase and 17,20-lyase activities of P450c17: contributions of serine106 and P450 reductase. *Endocrinology* **132**, 2498–2506.
- Lin, D., Harikrishna, J.A., Moore, C.C.D., Jones, K.L. and Miller, W.L. (1991) Missense mutation Ser106→Pro causes 17 $\alpha$ -hydroxylase deficiency. *J. Biol. Chem.* **266**, 15992–15998.
- Lin, D., Zhang, L., Chiao, E. and Miller, W.L. (1994) Modeling and mutagenesis of the active site of human P450c17. *Mol. Endocrinol.* **8**, 392–402.
- Matteson, K.J., Picado-Leonard, J., Chung, B., Mohandas, T.K. and Miller, W.L. (1986) Assignment of the gene for adrenal P450c17 (17 $\alpha$ -hydroxylase/17,20-lyase) to human chromosome 10. *J. Clin. Endocrinol. Metab.* **63**, 789–791.
- Miller, W.L. (1988) Molecular biology of steroid hormone synthesis. *Endocr. Rev.* **9**, 295–318.
- Miller, W.L. (1995) Mitochondrial specificity of the early steps in steroidogenesis. *J. Steroid Biochem. Molec. Biol.* **55**, 607–616.
- Miller, W.L. (1998) Early steps in androgen biosynthesis: from cholesterol to DHEA. *Bailliere's Clin. Endocrinol. Metab.* **12**, 67–81.
- Miller, W.L. (1999) The molecular basis of adrenarche -An hypothesis. *Acta Paed.* **88** (Suppl.), 60–66.
- Miller, W.L., Geller, D.H. and Auchus, R.J. (1998) The molecular basis of isolated 17,20-lyase deficiency. *Endocr. Res.* **24**, 817–825.
- Miura, K., Yasuda, K., Yanase, T., Yamakita, N., Sasano, H., Nawata, H., Inoue, M., Fukaya, T. and Shizuta, Y. (1996) Mutation of cytochrome P-45017 $\alpha$  gene (CYP17) in a Japanese patient previously reported as having glucocorticoid-responsive hyperaldosteronism: with a review of Japanese patients with mutations of CYP17. *J. Clin. Endocrinol. Metab.* **81**, 3797–3801.
- Monno, S., Mizushima, Y., Toyoda, N., Kashii, T. and Kobayashi, M. (1997) A new variant of the cytochrome P450c17 (CYP17) gene mutation in three patients with 17 $\alpha$ -hydroxylase deficiency. *Ann. Hum. Genet.* **61**, 275–279.
- Monno, S., Ogawa, H., Date, T., Fujioka, M., Miller, W.L. and Kobayashi, M. (1993) Mutation of histidine 373 to leucine in cytochrome P450c17 causes 17 $\alpha$ -hydroxylase deficiency. *J. Biol. Chem.* **268**, 25811–25817.
- Morales, A.J., Nolan, J.J., Nelson, J.C. and Yen, S.S.C. (1994) Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J. Clin. Endocrinol. Metab.* **78**, 1360–1367.
- Nakajin, S. and Hall, P.P. (1981a) Microsomal cytochrome P450 from neonatal pig testis. Purification and properties of a C21 steroid side-chain cleavage system (17 $\alpha$ -hydroxylase-C17,20-lyase). *J. Biol. Chem.* **256**, 3871–3876.

- Nakajin, S. and Hall, P.F. (1981b) Testicular microsomal cytochrome P450 for C21 steroid side-chain cleavage. *J. Biol. Chem.* **256**, 6134–6139.
- Nakajin, S., Shinoda, M. and Hall, P.F. (1983) Purification and properties of 17 $\alpha$ -hydroxylase from microsomes of pig adrenal: a second C21 side-chain cleavage system. *Biochem. Biophys. Res. Commun.* **111**, 512–517.
- Nakajin, S., Shinoda, M., Haniu, M., Shively, J.E. and Hall, P.F. (1984) C21 steroid side-chain cleavage enzyme from porcine adrenal microsomes. Purification and characterization of the 17 $\alpha$ -hydroxylase/C 17,20 lyase cytochrome P450. *J. Biol. Chem.* **259**, 3971–3976.
- Nakajin, S., Shively, J.E., Yuan, P. and Hall, P.F. (1981) Microsomal cytochrome P450 from neonatal pig testis: two enzymatic activities (17 $\alpha$ -hydroxylase and C 17,20-lyase) associated with one protein. *Biochemistry* **20**, 4037–4042.
- Nakajin, S., Takahashi, M., Shinoda, M. and Hall, P.F. (1985) Cytochrome b<sub>5</sub> promotes the synthesis of  $\Delta^{16}$ -C19 steroids by homogeneous cytochrome P-450 C21 side-chain cleavage from pig testis. *Biochem. Biophys. Res. Commun.* **132**, 708–713.
- Omura, T. and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239**, 2370–2378.
- Onoda, M. and Hall, P.F. (1982) Cytochrome b<sub>5</sub> stimulates purified testicular microsomal cytochrome P450 (C21 side-chain cleavage). *Biochem. Biophys. Res. Commun.* **108**, 454–460.
- Orentreich, N., Brind, J.L., Rizer, R.L. and Vogelmann, J.H. (1984) Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood. *J. Clin. Endocrinol. Metab.* **59**, 551–555.
- Ortiz de Montellano, P.R. (1986) In: *Cytochrome P-450: Structure, Mechanism and Biochemistry*, P.R. Ortiz de Montellano (ed), Plenum Press, New York, pp. 217–271.
- Oshiro, C., Takasu, N., Wakugami, T., Komiya, I., Yamada, T., Eguchi, Y. and Takei, H. (1995) Seventeen  $\alpha$ -hydroxylase deficiency with one base pair deletion of the cytochrome P450c17 (CYP17) gene. *J. Clin. Endocrinol. Metab.* **80**, 2526–2529.
- Picado-Leonard, J. and Miller, W.L. (1987) Cloning and sequence of the human gene encoding P450c17 (steroid 17 $\alpha$ -hydroxylase/17,20-lyase): similarity to the gene for P450c21. *DNA* **6**, 439–448.
- Picado-Leonard, J. and Miller, W.L. (1988) Homologous sequences in steroidogenic enzymes, steroid receptors and a steroid binding protein suggest a consensus steroid-binding sequence. *Mol. Endocrinol.* **2**, 1145–1150.
- Poulos, T.L., Finzel, B.C. and Howard, A.J. (1987) High-resolution crystal structure of cytochrome P450cam. *J. Mol. Biol.* **195**, 687–700.
- Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J. (1993) Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's. *Science* **261**, 731–736.
- Rumsby, G., Skinner, C., Lee, H.A and Honour, J.W. (1993) Combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency caused by heterozygous stop codons in the cytochrome P450 17 $\alpha$ -hydroxylase gene. *Clin. Endocrinol. (Oxf)* **39**, 483–485.
- Sakai, N., Tanaka, M., Adachi, S., Miller, W.L. and Nagahama, Y. (1992) Rainbow trout cytochrome P450c17 (17 $\alpha$ -hydroxylase/17,20 lyase) cDNA cloning, enzymatic properties and temporal pattern of ovarian P450c17 mRNA expression during oogenesis. *FEBS Lett.* **301**, 60–64.
- Sasano, H., Mason, J.I., Sasano, N. and Nagura, H. (1990) Immunolocalization of 3 $\beta$ -hydroxysteroid dehydrogenase in human adrenal cortex and its disorders. *Endocr. Pathol.* **1**, 94–101.
- Satoh, J., Kuroda, Y., Nawata, H. and Yanase, T. (1998) Molecular basis of hypokalemic myopathy caused by 17 $\alpha$ -hydroxylase/17,20-lyase deficiency. *Neurology* **51**, 1748–1751.

- Schulte, H.M. and Allolio, B. (1998) Oral dehydroepiandrosterone for adrenal androgen replacement: pharmacokinetics and peripheral conversion to androgens and estrogens in young healthy females after dexamethasone suppression. *J. Clin. Endocrinol. Metab.* **83**, 1928–1934.
- Sklar, C.A., Kaplan, S.L. and Grumbach, M.M. (1980) Evidence for dissociation between adrenarche and gonadarche: studies in patients with idiopathic precocious puberty, gonadal dysgenesis, isolated gonadotropin deficiency, and constitutionally delayed growth and adolescence. *J. Clin. Endocrinol. Metab.* **51**, 548–556.
- Smail, P.J., Faiman, C., Hobson, W.C., Fuller, G.B. and Winter, J.S.D. (1982) Further studies on adrenarche in nonhuman primates. *Endocrinology* **111**, 844–848.
- Sparkes, R.S., Klisak, I. and Miller, W.L. (1991) Regional mapping of genes encoding human steroidogenic enzymes: P450<sub>scc</sub> to 15q23-q24, adrenodoxin to 11q22; adrenodoxin reductase to 17q24-q25; and P450<sub>c17</sub> to 10q24-q25. *DNA Cell. Biol.* **10**, 359–365.
- Stevens, J.C., Jaw, J.Y., Peng, C.T. and Halpert, J. (1991) Mechanism-based inactivation of bovine adrenal cytochromes P450 C-21 and P450 17 $\alpha$  by 17 $\beta$ -substituted steroids. *Biochemistry* **30**, 3649–3685.
- Stocco, D.M. and Clark, B.J. (1996) Regulation of the acute production of steroids in steroidogenic cells. *Endocr. Rev.* **17**, 221–244.
- Suzuki, Y., Nagashima, T., Nomura, Y., Onigata, K., Nagashima, K. and Morikawa, A. (1998) A new compound heterozygous mutation (W17X, 436+5G→T) in the cytochrome P450<sub>c17</sub> gene causes 17 $\alpha$ -hydroxylase/17,20-lyase deficiency. *J. Clin. Endocrinol. Metab.* **83**, 199–202.
- Swart, P., Swart, A.C., Waterman, M.R., Estabrook, R.W. and Mason, J.I. (1993) Progesterone 16 $\alpha$ -hydroxylase activity is catalyzed by human cytochrome P45017 $\alpha$ -hydroxylase. *J. Clin. Endocrinol. Metab.* **77**, 98–102.
- Tomlinson, E.S., Lewis, D.F.V., Maggs, J.L., Kroemer, H.K., Park, B.K. and Back, D.J. (1997) *In vitro* metabolism of dexamethasone (DEX) in human liver and kidney: the involvement of CYP3A4 and CYP17 (17,20-lyase) and molecular modeling studies. *Biochem. Pharmacol.* **54**, 605–611.
- Voutilainen, R., Tapanainen, J., Chung, B., Matteson, K.J. and Miller, W.L. (1986) Hormonal regulation of P450<sub>scc</sub> (20, 22-desmolase) and P450<sub>c17</sub> (17 $\alpha$ -hydroxylase/17,20-lyase) in cultured human granulosa cells. *J. Clin. Endocrinol. Metab.* **63**, 202–207.
- Yamaguchi, H., Nakazato, M., Miyazato, M., Kangawa, K. and Matsukura, S. (1997) A 5'-splice site mutation in the cytochrome P450 steroid 17 $\alpha$ -hydroxylase gene in 17 $\alpha$ -hydroxylase deficiency. *J. Clin. Endocrinol. Metab.* **82**, 1934–1938.
- Yanagibashi, K. and Hall, P.F. (1986) Role of electron transport in the regulation of the lyase activity of C-21 side-chain cleavage P450 from porcine adrenal and testicular microsomes. *J. Biol. Chem.* **261**, 8429–8433.
- Yanase, T. (1995) 17 $\alpha$ -Hydroxylase/17,20-lyase defects. *J. Steroid Biochem. Mol. Biol.*, **53**, 153–157.
- Yanase, T., Kagimoto, M., Matsui, N., Simpson, E. and Waterman M.R. (1988) Combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency due to a stop codon in the N-terminal region of 17 $\alpha$ -hydroxylase cytochrome P-450. *Mol. Cell. Endocrinol.* **59**, 249–253.
- Yanase, T., Kagimoto, M., Suzuki, S., Hashiba, K., Simpson, E.R. and Waterman, M.R. (1989) Deletion of a phenylalanine in the N-terminal region of human cytochrome P-45017 $\alpha$  results in the partial combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency. *J. Biol. Chem.* **264**, 18076–18082.
- Yanase, T., Sanders, D., Shibata, A., Matsui, N., Simpson, E.R. and Waterman, M.R. (1990) Combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency due to a 7-basepair duplication in the N-terminal region of the cytochrome P45017 $\alpha$  (CYP17) gene. *J. Clin. Endocrinol. Metab.* **70**, 1325–1329.

- Yanase, T., Sasano, H., Yubisui, T., Sakai, Y., Takayanagi, R. and Nawata, H. (1998) Immunohistochemical study of cytochrome b<sup>5</sup> in human adrenal gland and in adrenocortical adenomas from patients with Cushing's syndrome. *Endocr. J.* **45**, 89–95.
- Yanase, T., Simpson, E.R. and Waterman, M.R. (1991) 17 $\alpha$ -hydroxylase/17,20-lyase deficiency: from clinical investigation to molecular definition. *Endocr. Rev.* **12**, 91–108.
- Yanase, T., Waterman, M.R., Zachmann, M., Winter, J.S.D., Simpson, E.R. and Kagimoto, M. (1992) Molecular basis of apparent isolated 17,20-lyase deficiency: compound heterozygous mutations in the C-terminal region (Arg(496)→Cys, Gln(461)→Stop) actually cause combined 17 $\alpha$ -hydroxylase/17,20-lyase deficiency. *Biochim. Biophys. Acta* **1139**, 275–279.
- Yasukochi, Y. and Masters, B.S. (1976) Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**, 5337–5344.
- Zachmann, M., Kenpken, B., Manella, B. and Navarro, E. (1992) Conversion from pure 17,20-desmolase to combined 17,20-desmolase/17 $\alpha$ -hydroxylase deficiency with age. *Acta Endocrinol.* **127**, 97–99.
- Zachmann, M., Vollmin, J.A., Hamilton, W. and Prader, A. (1972) Steroid 17,20-desmolase deficiency: a new cause of male pseudohermaphroditism. *Clin. Endocrinol.* **1**, 369–385.
- Zachmann, M., Werder, E.A. and Prader, A. (1982) Two types of male pseudohermaphroditism due to 17,20 desmolase deficiency. *J. Clin. Endocrinol. Metab.* **55**, 487–490.
- Zhang, L., Rodriguez, H., Ohno, S. and Miller, W.L. (1995) Serine phosphorylation of human P450c17 increases 17,20-lyase activity: implications for adrenarche and for the polycystic ovary syndrome. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10619–10623.
- Zuber, M.X., John, M.E., Okamura, T., Simpson, E.R. and Waterman, M.R. (1986a) Bovine adrenal cytochrome P45017: regulation of gene expression by ACTH and elucidation of primary sequence. *J. Biol. Chem.* **261**, 2475–2482.
- Zuber, M.X., Simpson, E.R. and Waterman, M.R. (1986b) Expression of bovine 17 $\alpha$ -hydroxylase cytochrome P450 cDNA in non-steroidogenic (COS-1) cells. *Science* **234**, 1258–1261.



## 10.

# AROMATASE: INSIGHTS INTO THE ROLES OF ESTROGENS REVEALED BY NATURAL AND TARGETED MUTATIONS

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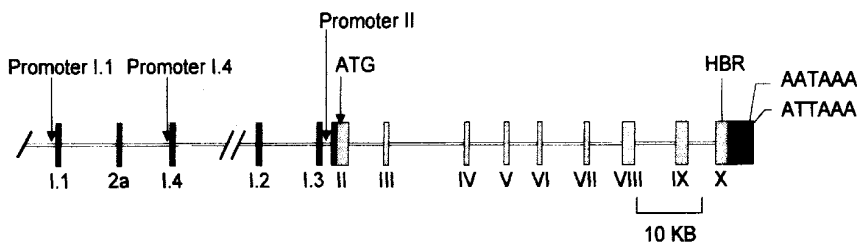
Physiological estrogens are formed from androgenic steroids by the action of the enzyme aromatase, which is a member of the cytochrome P450 superfamily, and encoded by the CYP19 gene. In humans, aromatase expression and hence estrogen biosynthesis occurs in a number of tissue sites including ovary, placenta, fetal liver, adipose, bone and brain. Tissue-specific expression is regulated by tissue-specific promoters which are located upstream of a number of untranslated first exons which are incorporated into the transcripts by alternative splicing. Analysis of the phenotypes of humans with natural mutations of the aromatase gene, as well as mice in which the gene has been inactivated by targeted disruption, have revealed a number of new and unexpected roles for estrogens in both males and females. These include roles in bone mineralization, lipid and carbohydrate metabolism, and spermatogenesis in the male. This latter role questions the very definition of the term "estrogen", at least in mice, since in this limited context, estradiol should more properly be considered androgenic. This review will briefly summarise the present and sometimes contradictory state of knowledge regarding the diverse roles which are played by the products of the aromatase reaction, and point to future directions which may help to clarify these issues.

## INTRODUCTION

Estrogen biosynthesis is catalysed by a microsomal member of the cytochrome P450 superfamily, namely aromatase cytochrome P450 (CYP19, also known as P450arom, the product of the CYP19 gene). The P450 gene superfamily is a very large one, containing over 600 members in almost 100 families, of which CYP19 is the sole member of family 19. This heme protein is responsible for binding of the C<sub>19</sub>-androgenic steroid substrate and catalysing the series of reactions leading to formation of the phenolic A ring characteristic of estrogens. The aromatase reaction employs 3 moles of oxygen and 3 moles of NADPH for every mole of steroid substrate metabolised (Thompson and Siiteri, 1974). These oxygen molecules are utilized in oxidation of the C<sub>19</sub> angular methyl group to formic acid, which occurs concomitantly with aromatisation of the A ring to give the phenolic A ring characteristic of estrogens (Oh and Robertson, 1993). The reducing equivalents for this

reaction are supplied from NADPH via a ubiquitous microsomal flavoprotein, NADPH-cytochrome P450 reductase. In humans, a number of tissues have the capacity to express aromatase and hence synthesise estrogens. These include the ovaries and testes, the placenta and fetal (but not adult) liver, adipose tissue, chondrocytes and osteoblasts of bone, and numerous sites in the brain including several areas of the hypothalamus, limbic system and cerebral cortex.

The human CYP19 gene was cloned some years ago (Means *et al.*, 1989; Harada *et al.*, 1990; Toda *et al.*, 1990), when it was shown that the coding region spans 9 exons beginning with exon II. Upstream of exon II are a number of alternative exons I which are spliced in the 5'-untranslated region of the transcript in a tissue-specific fashion (Figure 10.1). Thus placental transcripts contain at their 5'-end a distal exon, I.1, which is localised at least 40kb upstream from the start of translation in exon II. This is because placental expression is driven by a powerful distal promoter upstream of exon I.1 (Means *et al.*, 1991). On the other hand, transcripts in ovary and testes contain at their 5'-end, sequence that is immediately upstream of the translational start site. This is because expression of the gene in the gonads utilizes a proximal promoter, promoter II (Jenkins *et al.*, 1993). By contrast, transcripts in adipose tissue contain yet another distal exon located at least 20kb upstream of the start of translation, exon I.4 (Mahendroo *et al.*, 1993). A number of other untranslated exons have been characterized including one which is expressed in brain (Honda *et al.*, 1994). Splicing of these untranslated exons to form the mature transcript occurs at a common 3'-splice junction that is upstream of the translational start site. This means that although transcripts in different tissues have different 5'-termini, the coding region and thus the protein expressed in these various tissue sites is always the same. However, the promoter regions upstream of each of the several untranslated first exons have different cohorts of response elements, and so regulation of aromatase expression in each tissue that synthesises estrogens is different. Thus, the gonadal promoter binds the transcription factors CREB and SP1, and so aromatase expression in gonads is regulated by cAMP and gonadotropins. On the other hand, the adipose promoter, I.4, is regulated by class I cytokines such as IL-6, IL-11 and oncostatin M, as well as by TNF $\alpha$ . Thus the regulation of estrogen biosynthesis in each tissue site of expression is unique (reviewed in Simpson *et al.*, 1997).

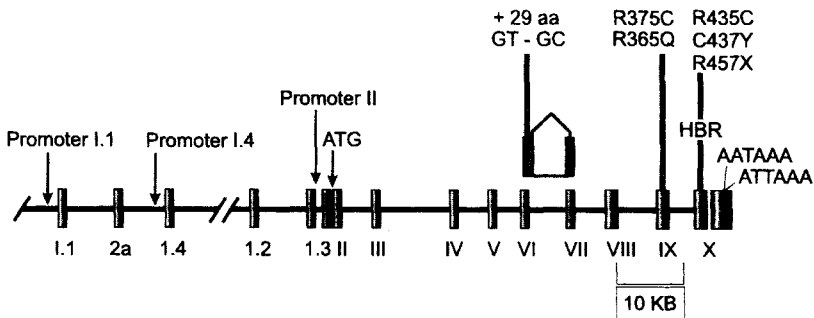


**Figure 10.1** Diagram of the structure of the human aromatase gene showing the arrangement of the various untranslated first exons and their associated promoters.

# AROMATASE DEFICIENCY IN HUMANS

Aromatase deficiency appears to be a rare condition and there have been ten mutations reported to date, affecting seven females and three males, one of whom is a child (Figure 10.2) (Shozu *et al.*, 1991; Ito *et al.*, 1993; Morishima *et al.*, 1995; Portrat-Doyen *et al.*, 1996; Carani *et al.*, 1997; Mullis *et al.*, 1997). The phenotypes of these individuals will be summarized in this presentation as will the findings on the phenotype of aromatase knockout mice (ArKO) created by disruption of the CYP19 gene which encodes aromatase.

With the exception of the first reported case, namely the Japanese patient (Shozu *et al.*, 1991), all of the mutations so far identified are single base-pair changes giving rise to single amino acid substitutions and in one case a premature stop-codon (Carani *et al.*, 1997) (Figure 10.2). The mutation in the Japanese patient is a single base change that destroyed an exon-intron splice junction, giving rise to continued read through of an extra 87 bases to a cryptic intron-exon splice junction within the intron, resulting in an in-frame insertion of 29 amino acids within the coding region. It should be pointed out that both siblings in the New York family (Morishima *et al.*, 1995) were homozygous for the condition, which would suggest that the mutation does not cause any diminished likelihood of implantation, nor any serious problem with embryonic or fetal development. Apart from the Californian patient and the Swiss patient who are compound heterozygotes (Ito *et al.*, 1993), all of the subjects are homozygous for the mutation in question, and are the products of consanguineous relationships. In most cases it was the mother who presented, during the third trimester, complaining of virilization resulting in facial hair and acne. These symptoms subsided after delivery. In the cases of the female newborns, they present with pseudohermaphroditism with clitoromegaly and hypospadias in varying degrees of severity. This virilization of both mother and fetus is a consequence of the inability of DHEA of fetal adrenal origin to be converted to estrogens by the placenta, with its consequent peripheral conversion to androgens. These



**Figure 10.2 Known mutations in the CYP19 gene that give rise to estrogen insufficiency in humans.** For details see references (Shozu *et al.*, 1991; Ito *et al.*, 1993; Morishima *et al.*, 1995; Portrat-Doyen *et al.*, 1996; Carani *et al.*, 1997). The mutations in the Swiss compound heterozygote case (Mullis *et al.*, 1997) are not included.

individuals then present again at the time of puberty with primary amenorrhea, failure of breast development, hypergonadotrophic-hypogonadism and cystic ovaries. Subsequent estrogen supplementation leads to regression of these symptoms.

**Table 10.1** Plasma hormone levels in the male patient with CYP19 deficiency.

$\Delta^4\text{A}$	335	ng/dl	30–263
T	2015	ng/dl	200–1200
5 $\alpha$ -DHT	125	ng/dl	30–85
E1	<7	pg/ml	10–50
E2	<7	pg/ml	10–50
FSH	28.3	mIU/ml	5.0–9.9
LH	26.1	mIU/ml	2.0–9.9
Glucose	70	mg/dl	70–105
Insulin	52	$\mu\text{U/ml}$	5–25
GH	<0.5	ng/ml	0.5–4.2
IGF-1	203	ng/ml	182–780

In addition two men have been reported with this condition (Morishima *et al.*, 1995; Carani *et al.*, 1997). In each case childhood development was uneventful. Each presented in their late twenties with tall stature due to sustained linear growth through puberty as a consequence of failure of epiphyseal fusion. They also had severely delayed bone age resulting in osteopenia and under-mineralization.

Analysis of the plasma hormone levels of the New York male patient (Table 10.1) revealed undetectable estrogens and very high circulating androgens (Morishima *et al.*, 1995). Circulating FSH and LH were also elevated, indicative of an important role for estrogens in the negative feedback regulation of gonado-trophins in males as in females. Presumably, in the case of males, this estrogen is normally derived from local aromatization of testosterone within brain sites. The patient was 204cm tall at the age of 24 years and had testes which were 35ml in volume. Unfortunately he declined to give a semen sample so it was impossible to gauge his sperm count or sperm viability. He was unmarried and had no offspring. However his testicular volume and hormonal profile would indicate the likelihood that he had functioning testes as far as their capacity to synthesize steroids was concerned.

The fetal testes synthesizes anti-Mullerian hormone (AMH) and testosterone, the former causing regression of the Mullerian structures and the latter causing virilization of the male external genitalia (Figure 10.3). Classically this action of testosterone is thought to be achieved by conversion to 5 $\alpha$ -dihydrotestosterone due to the activity of 5 $\alpha$ -reductase. However, more recently it has become evident that there are several sites in the testes which are capable of converting testosterone to estrogens. This is because the enzyme aromatase, responsible for the conversion of androgens to estrogens, has been reported to be present at a number of sites within the testes including the Leydig cells, but also germ cells in various stages of development (Nitta *et al.*, 1993; Janulis *et al.*, 1996). Additionally, both the  $\alpha$  and  $\beta$  isoforms of the estrogen receptor have been reported to be present at several sites (Fisher *et al.*, 1997; Kuiper *et al.*, 1997; Saunders *et al.*, 1998). Furthermore, the levels of estradiol in the semen and rete fluid are greater than those circulating in the plasma of females (Ganiam and Amann, 1976; Free and Jaffe, 1979). These considerations give rise to the concept that estrogens play a role in the function of the testes.

The second male patient was from a family in southern Italy who presented at the age of 28 years with tall stature, infertility and skeletal pain. He was found to have open epiphyses and a bone age of 14.8 years. Treatment with testosterone for 8 months failed to result in

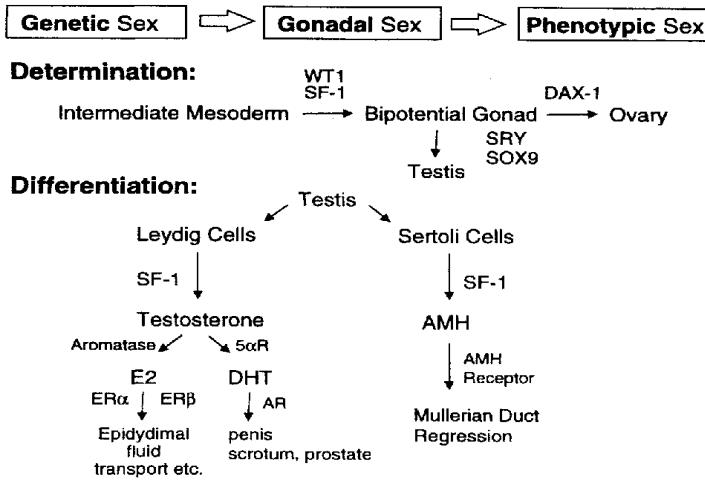


Figure 10.3 Factors involved in testicular differentiation.

any improvement of his condition, whereas treatment with transdermal estradiol for 6 months restored his bone density to within the normal range and eliminated his other symptoms. In contrast to the New York patient, this individual had a testicular volume of 8ml and was infertile. Testicular biopsy revealed that his seminiferous tubules had little or no sperm present and gamete development appeared to be arrested at the spermatocyte level. In contrast to the New York patient again, his circulating testosterone levels were not elevated and were reduced dramatically upon administration of estradiol. These results might suggest a relationship between aromatase deficiency and testicular dysfunction. Unfortunately for this concept though, the individual has a brother with azoospermia but who is homozygous for the normal active aromatase gene. These observations suggest that the fertility problem in these men may be independent of the aromatase deficiency in this family. Thus the study of these two men with aromatase deficiency has failed to elucidate the role of estrogens in male fertility. The phenotypes of these men may be compared with that of the one known male with a mutation in the estrogen receptor  $\alpha$  isoform, who also presented with a failure of epiphyseal closure and undermineralized bones, but who has reduced sperm count and sperm motility (Smith *et al.*, 1994).

In addition to the bone and fertility phenotypes outlined above, individuals with aromatase deficiency have a lipid and carbohydrate phenotype, namely truncal obesity, hypertriglyceridemia, elevated LDL and insulin resistance. These symptoms all subside upon treatment with estrogen (Table 10.1).

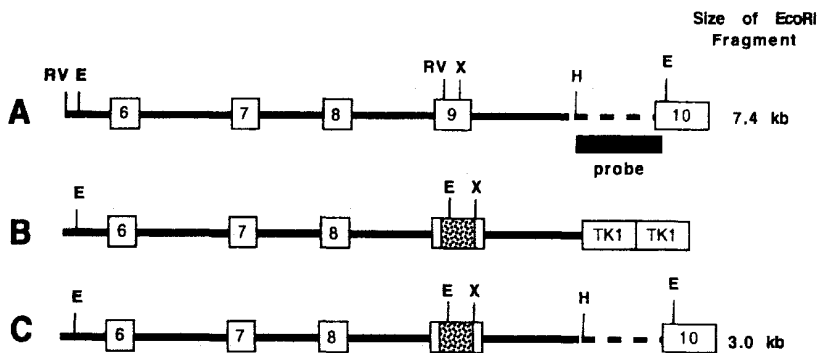
### THE AROMATASE KNOCKOUT (ArKO) MOUSE

In order to gain further insight into the physiological role of estrogens, a mouse was created in which the aromatase gene was disrupted by homologous recombination (Figure 10.4) (Fisher *et al.*, 1998). Studies of these mice are indicative of a dramatic bone phenotype, with

shortened femur length and reduction in all of the indices of bone mineralization. Because circulating estradiol levels in wild-type as well as ArKO male mice are below the level of detection, this would indicate that the source of estrogens maintaining bone mineralization in the wild-type mice is likely to be local production within the bone itself or at other extragonadal sites.

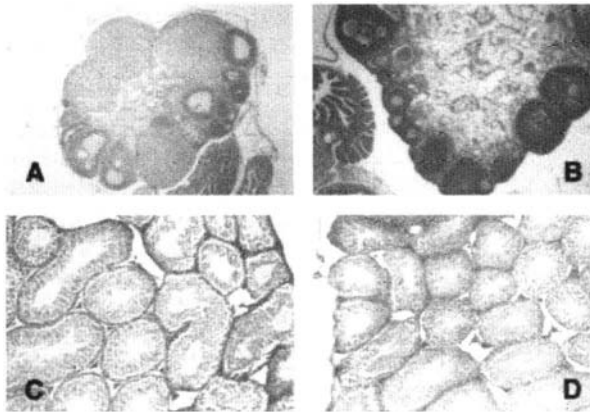
Examination of the morphology of the ovaries of mice at the age of 10–12 weeks (Figure 10.5) revealed a striking phenotype in that the ovaries contained a number of follicles which showed signs of antral information but appeared arrested prior to ovulation. No corpora lutea were present but the follicles had abundant granulosa cells displaying some mitotic figures. The stroma was hyperplastic, indicative of the high circulating LH levels and some pyknotic figures were present, perhaps indicative of follicular atresia. At later ages cystic hemorrhagic follicles were present, coincident with an infiltration of macrophages and collagen deposition in the interstitium. By one year of age there were no secondary or antral follicles in ArKO ovaries and atresia was widespread in the remaining primary follicles.

Examination of the testes of male mice at a similar age showed no obvious phenotypes and these males were fertile (Figure 10.5). However with increasing



**Figure 10.4** Diagram of part of the structure of the wild-type mouse CYP19 gene (A), the targeting vector (B), and the disrupted gene (C).

age, the numbers of litters they sired diminished as compared to their wild-type littermates. Disruptions to spermatogenesis commenced at 4–5 months and became more progressive with advancing age (Robertson *et al.*, 1999). Spermatogenesis was primarily arrested at early spermiogenic stages, as characterized by an increase in apoptosis and the appearance of multinucleated cells, and there was a significant reduction in round and elongated spermatids, but no changes in Sertoli cells and earlier germ cells. In addition, Leydig cell hyperplasia/hypertrophy was evident, presumably as a consequence of increased circulating LH. These findings suggest that local expression of aromatase, at least in mice, is essential for spermatogenesis and provide evidence for a direct action of estrogen on male germ cell development and thus, fertility. These results are in contrast to those of the ER $\alpha$ -knockout mice (ERKO) in which the seminiferous tubules had grossly distended lumens with no sign of sperm (Lubahn *et al.*, 1993). This is apparently due to failure of fluid transfer across the epithelium of the efferent ductules (Hess *et al.*, 1997), resulting in increased pressure and back-up of fluid into the seminiferous tubules. By contrast, the ER $\beta$ -knock-out mice display



**Figure 10.5** Optical micrographs of ovaries (A and B) and testes (C and D) of wild-type (A and C) and ArKO (B and D) mice at 12–14 weeks of age. Magnification: A,  $\times 10$ ; B–D,  $\times 20$ . (See [color plate 2](#))

no detectable testicular phenotype. These differences in phenotype of the various models of lack of estrogen representation are not readily explicable in terms of our current level of understanding. There are several possibilities, including: the presence of endogenous estrogenic substances other than the products of the aromatase reaction; non-ligand-mediated pathways of estrogen receptor activation; and pathways of estrogen action other than via the known receptors.

Another phenotype developed by these mice, similar to the situation with humans carrying a natural mutation in the CYP19 gene, is a lipid and carbohydrate phenotype. ArKO mice, both male and female, develop a progressive increase in abdominal adiposity, involving both the gonadal and infra-renal fat deposits. This is associated with increased plasma triglycerides and cholesterol, insulin resistance and hyperleptinemia. Hepatic steatosis is also present. Interestingly, the increased adiposity is not accompanied by a marked increase in body weight. This is because there is a corresponding decrease in lean body mass, most likely skeletal muscle, which is associated with a decrease in activity. This adiposity phenotype is reversed dramatically within three weeks upon administration of estradiol in the form of implants. A similar phenotype has been reported for the ER $\alpha$ KO mice, so these two models are in accordance as far as this phenotype is concerned.

## CONCLUSIONS

Models of estrogen insufficiency, whether they be natural mutations in humans or targeted gene disruptions in mice, are revealing new and often unsuspected roles for estrogens in both males and females. In some cases consistency is observed between the animal models, such as the adipose phenotype of the ArKO and ER $\alpha$ KO mice. On the other hand, the studies conducted so far on aromatasedeficient humans have not served to throw light on the role of estrogens in male reproduction. This is because of the conflicting phenotypes of the

two men with aromatase deficiency, as well as the apparent discrepancies between the phenotype of the ArKO mice and that of the ER $\alpha$ KO and ER $\beta$ KO mice. This issue can only be resolved by more extensive characterization of the phenotypes of these animals at the cellular and molecular level. There is also a pressing need to identify another man with aromatase deficiency, since the observations on the two known men do not permit unambiguous answers.

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#### REFERENCES

- Carani, C., Qin, K., Simoni, M., Fanstini-Fustini, M., Serpente, S., Boyd, J., Korach, K.S. and Simpson, E.R. (1997) Effect of testosterone and estradiol in a man with aromatase deficiency. *N. Eng. J. Med.* **337**, 91–95.
- Fisher, C.R., Graves, K.H., Parlow, A.F. and Simpson, E.R. (1998) Characterization of mice deficient in aromatase (ArKO) due to targeted disruption of the GYP-19 gene. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6965–6970.
- Fisher, J., Millar, M.R., Majdic, G., Saunders, P.T.K., Fraser, H.M. and Sharpe, R.M. (1997) Immunolocalization of oestrogen receptor alpha within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *J. Endocrinol.* **153**, 485–495.
- Free, M.J. and Jaffe, R.A. (1979) Collection of rete testis fluid from rats without previous efferent duct ligation. *Biol. Reprod.* **20**, 269–278.
- Ganiam, V.K. and Amann, R.P. (1976) Steroid content of fluids and sperm entering and leaving the bovine epididymis, in epididymal tissue, and in accessory sex gland secretions. *Endocrinology* **99**, 1618–1630.
- Harada, N., Yamada, K., Saito, K., Kibe, N., Dohmae, S. and Takagi, Y. (1990) Structural characterization of the human estrogen synthetase (aromatase) gene. *Biochem. Biophys. Res. Commun.* **166**, 365–372.
- Hess, R.A., Bunick, D., Lee, K.-H., Bahr, J., Taylor, J.A., Kovach, K.S. and Lubahn, D.B. (1997) A role for oestrogens in the male reproductive system. *Nature* **390**, 509–512.
- Honda, S., Harada, N. and Takagi, Y. (1994) Novel exon I of the aromatase gene specific for aromatase transcripts in human brain. *Biochem. Biophys. Res. Commun.* **198**, 1153–1160.
- Ito, Y., Fisher, C.R., Conte, F.A., Grumbach, M.M. and Simpson, E.R. (1993) The molecular basis of aromatase deficiency in an adult female with sexual infantilism and polycystic ovaries. *Proc. Natl. Acad. Sci. U.S.A.* **9**, 1163–1167.
- Janulis, L., Bahr, J.M., Hess, R.A. and Bunick, D. (1996) P450 aromatase messenger ribonucleic acid and expression in male germ cells: detection by reverse-transcription-polymerase chain reaction. *J. Androl.* **17**, 651–658.
- Jenkins, C., Michael, D., Mahendroo, M. and Simpson, E.R. (1993) Exon-specific northern analysis and rapid amplification of cDNA ends (RACE) reveal that the proximal promoter II is responsible for aromatase cytochrome P450 expression in human ovary. *Mol. Cell. Endocrinol.* **97**, R1–R6.



- Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilson, S. and Gustafsson, J.A. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863–870.
- Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S. and Smithies, O. (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11162–11166.
- Mahendroo, M., Mendelson, C.R. and Simpson, E.R. (1993) Tissue-specific and hormonally controlled promoters regulate aromatase cytochrome P450 gene expression in human adipose tissue. *J. Biol. Chem.* **268**, 19463–19470.
- Means, G.D., Kilgore, M.W., Mahendroo, M., Mendelson, C.R. and Simpson, E.R. (1991) Tissue-specific promoters regulate aromatase cytochrome P450 expression in human ovary and fetal tissues. *Mol. Endocrinol.* **5**, 2005–2013.
- Means, G.D., Mahendroo, M., Corbin, C.J., Mathis, J.M., Powell, F.E., Mendelson, C.R. and Simpson, E.R. (1989) Structural analysis of the gene encoding human aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *J. Biol. Chem.* **264**, 19385–19391.
- Morishima, A., Grumbach, M.M., Simpson, E.R., Fisher, C. and Kenan, Q. (1995) Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J. Clin. Endocrinol. Metab.* **8**, 3689–3698.
- Mullis, P.E., Yoshimura, N., Kuhlmann, B., Lippuner, K., Jaeger, P. and Harada, N. (1997) Aromatase deficiency in a female who is a compound heterozygote for two new point mutations in the P450arom gene: impact of estrogens on hypergonadotropic hypogonadism, multicystic ovaries, and bone density in childhood. *J. Clin. Endocrinol. Metab.* **82**, 1739–1745.
- Nitta, H., Bunick, D., Hess, R.A., Janulis, L., Newton, S.C., Millette, C.F., Osawa, Y., Shizuta, Y., Toda, K. and Bahr, J.M. (1993) Germ cells of the mouse testis express P450 aromatase. *Endocrinology* **132**, 1396–1401.
- Oh, S.S. and Robinson, C.H. (1993) Mechanism of human placental aromatase: a new active site model. *J. Steroid Biochem. Molec. Biol.* **44**, 389–397.
- Portrat-Doyen, F., Forest, M.G., Nicolino, M., Morel, Y. and Chatelain, P.G. (1996) Female pseudohermaphroditism (FPH) resulting from aromatase (P450arom) deficiency associated with a novel mutation (R457) in the CYP-19 gene. *Horm. Res.* **46** (Suppl.), 14.
- Robertson K.M., O'Donnell, L., Jones M.E., Meachem, S.J., Boon, W.-C., Fisher, C.R., Graves, K.H., McLachlan, R.I. and Simpson, E.R. (1999). Impairment of spermatogenesis in mice lacking a functional aromatase (CYP19) gene. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7986–7991.
- Saunders, P.T.K., Fisher, J.S., Sharpe, R.M. and Millar, M.R. (1998) Expression of oestrogen receptor beta occurs in multiple cell types, including some germ cells, in the rat testis. *J. Endocrinol.* **156**, R13–R17.
- Shozu, M., Akasofu, K., Harada, T. and Kubota, Y. (1991) A new cause of female pseudohermaphroditism: placental aromatase deficiency. *J. Clin. Endocrinol. Metab.* **72**, 560–566.
- Simpson, E.R., Zhao, Y., Agarwal, V.R., Michael, M.D., Bulun, S.E., Hinshelwood, M.M., Graham-Lorence, S., Sun, T., Fisher, C.R., Qin, K. and Mendelson, C.R. (1997) Aromatase expression in health and disease. *Rec. Prog. Horm. Res.* **52**, 185–214.
- Smith, E.P., Boyd, J., Frank, G.R., Takahashi, H., Coben, R.M., Specker, B., Williams, T.C., Lubahn, D.B. and Korach, K.S. (1994) Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N. Engl. J. Med.* **331**, 1056–1061.
- Thompson, E.A. and Siiteri, P.K. (1974) Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatisation of androstenedione. *J. Biol. Chem.* **249**, 5364–5372.

- Toda, K., Terashima, M., Kamamoto, T., Sumimoto, H., Yokoyama, Y., Kuribayashi, I., Mitsuuchi, Y., Maeda, T., Yamamoto, Y., Sagara, Y., Ikeda, H. and Shizuta, Y. (1990) Structural and functional characterization of the human aromatase P450 gene. *Eur. J. Biochem.* **193**, 559–563.

# 11.

## 17 $\beta$ -HYDROXYSTEROID DEHYDROGENASE AND 5 $\alpha$ -REDUCTASE DEFICIENCIES

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The focus of this chapter will be to describe two enzymatic defects causing male pseudohermaphroditism namely, 17 $\beta$ -hydroxysteroid dehydrogenase/ketosteroid (17 $\beta$ -HSD/KSR) and 5 $\alpha$ -reductase deficiencies. Although these autosomal recessive disorders are rare, the study of their associated phenotypic characteristics is important for understanding of the mechanism of androgen action, the process of sexual differentiation and the factors that may influence normal sexual behavior. 17 $\beta$ -HSD/KSR deficiency is caused by impairment in the testicular conversion of  $\Delta^4$ -androstenedione ( $\Delta^4$ -DIONE) to testosterone (T), resulting from mutations in the HSD17B3 gene encoding type 3 17 $\beta$ -HSD/KSR. Most affected 46XY individuals have external female genitalia or a slightly enlarged clitoris, although their internal genitalia are well masculinized. The relatively normal masculinization of internal structures may result from the activity of another 17 $\beta$ -HSD/KSR isoenzyme(s) in the proximity of the Wolffian ducts, which ensures adequate local production of T from testis-derived  $\Delta^4$ -DIONE during the appropriate period of fetal life. However, considerable virilization of the external genitalia occurs at the time of puberty, which is also associated with development of male secondary sexual characteristics, although breast enlargement was observed at puberty in half of the patients. This apparent “recovery” in 17 $\beta$ -HSD/KSR activity results from the action of another 17 $\beta$ -HSD/KSR enzyme in peripheral tissues. Usually, the diagnosis of type 3 17 $\beta$ -HSD/KSR deficiency is made when a 10- to 15-fold elevation in the ratios of  $\Delta^4$ -androstenedione to T is observed. To date a total of 24 mutations (including two frameshift, four splicing and 18 missense mutations) have been identified in the HSD17B3 gene in approximately 46 distinct families. Among the 12 missense mutations that have been functionally characterized, only four mutations were found to retain a residual type 3 17 $\beta$ -HSD/KSR activity.

On the other hand, 5 $\alpha$ -reductase deficiency results from a blockade in the testicular conversion of T to dihydrotestosterone (DHT), caused by mutations in the SRD5A2 gene encoding 5 $\alpha$ -reductase type 2. This form of male pseudohermaphroditism in infancy includes clitoris-like hypospadiac phallus bound in chordee of variable degree, a bifid scrotum, and a urogenital sinus that opens on the perineum. The prostate is hypoplastic. Furthermore, in these affected individuals, like those affected by 17 $\beta$ -HSD/KSR deficiency, the normal testes are located in the inguinal canal or the labioscrotal folds, the epididymes,

vas deferens and seminal vesicles are well differentiated and usually terminate in the blind-ending vagina, whereas the Mullerian ducts normally regress. The diagnosis can be confirmed prepubertally or postpubertally by an abnormal plasma T/DHT ratio before or after hCG stimulation. At puberty, plasma T levels increase into adult range, while DHT levels remain disproportionately low but measurable. They exhibit at puberty signs of virilization with descent of the testes, muscle mass increases, decreased facial and body hair, no temporal hair recession and acne as well as deepening of the voice without gynecomastia. To date, a total of 38 mutations (including a complete deletion of the SRD5A2 gene, four frameshift, one splicing, 30 missense, two nonsense mutations) have been identified in the SRD5A2 gene in approximately 76 distinct families. Among the 22 missense mutations that have been functionally characterized, ten mutations were found to retain a residual activity. It is difficult to associate the molecular diagnosis with the observed phenotypic variability in patients suffering from 17 $\beta$ -HSD/KSR and 5 $\alpha$ -reductase deficiencies. Note that those females who have been identified to carry HSD17B3 or SRD5A2 deleterious mutations were all asymptomatic. Finally, the functional characterization of missense mutations known to be involved in these disorders also provides valuable information concerning the structure-function relationships of these enzyme families.

**KEY WORDS:** 17 $\beta$ -hydroxysteroid dehydrogenase deficiency, 5 $\alpha$ -reductase deficiency, male pseudohermaphroditism, molecular diagnosis, steroidogenesis, androgens.

## INTRODUCTION

The complex sequential process of normal sex determination and differentiation is regulated by at least 50 different genes located on sex chromosomes and autosomes that act through distinct mechanisms, including organizing factors, gonadal steroid and peptide hormones, and target-specific tissue receptors (Grumbach and Conte, 1999). In brief, normal sex development consists of 3 sequential processes (Forest, 1995), the first being the genetic sex determination by the sex chromosome constitution in the zygote at the time of conception. Thereafter, the genetic information determines whether an undifferentiated gonad differentiates into either a testis or an ovary (gonadal or primary sex). Finally, the phenotypic sex results in male differentiation, an active process resulting from testicular secretions. Among these hormones, the anti-Müllerian hormone (AMH) produced by the Sertoli cells, inhibits the Müllerian ducts, whereas testosterone (T) produced by the Leydig cells, is responsible for stabilization of the Wolffian ducts, and via its transformation into dihydrotestosterone (DHT), is also responsible for virilization of the external genitalia. Consequently, the genital primordia are irreversibly committed to the female state in the absence of testis (Forest, 1995).

Based on the recognition of the underlying anomaly, abnormalities of sexual development can be classified into two broad categories: (A) disorders of sex determination that are most

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often caused by sex chromosome or gene abnormalities that affect gonadogenesis. These abnormalities are mainly detectable by cytogenetic analysis or by using DNA probes for genes located on the Y chromosome; and (B) abnormal genital differentiation, which is usually caused by a genetic defect, or less commonly, by adverse factors in the intrauterine environment. For example, defects in the synthesis or action of androgens impair the development of the male phenotype during embryogenesis and cause the disorder of human intersex termed male pseudohermaphroditism (Forest *et al.*, 1995; Grumbach and Conte, 1999). The focus of this chapter will be to describe two enzymatic defects causing male pseudohermaphroditism namely,  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD) type 3 and  $5\alpha$ -reductase type 2 deficiencies. Although these enzymatic disorders are rare, the study of their associated phenotypic characteristics is important for understanding of the mechanism of androgen action, the process of sexual differentiation and the factors that may influence normal sexual behavior.

### Role of androgens in sex differentiation

In order to better understand the consequences of these two enzymatic disorders in the development of male phenotype, it appears useful to briefly summarize the role of T and DHT action in this complex process. The actions of T are varied and tissue specific and reflect the sum of its direct action and the effects of its  $5\alpha$ -reduced product, DHT as well as those of its aromatized product, estradiol. Although, T and DHT bind to the same high-affinity androgen receptor, they achieve distinct physiological roles. Indeed, T binds less avidly to AR than DHT, mainly as the consequence of a slower dissociation rate, whereas DHT-receptor complex is more readily transformed to the DNA-binding state and is thus more stable. The T-AR complex appears to play a major role in the stimulation of Wolffian ducts during sexual differentiation, and possibly in the control of spermatogenesis. However, the DHT-AR complex is responsible for external virilization during embryogenesis and most androgen-mediated events of male sexual maturation at puberty, namely growth of facial and body hair, temporal regression of scalp hair and maturation of the external genitalia (Wilson *et al.*, 1993).

The internal genital tracts in males and females arise from different anlagen. In the male, the Wolffian ducts that lack  $5\alpha$ -reductase activity give rise to epididymides, vasa deferentia and seminal vesicles, while the Müllerian ducts normally regress in response to anti-Müllerian hormone formed by the testicular Sertoli cells. This virilization process is completed at about 13 weeks of gestation, before the capacity to form DHT is acquired by these tissues. Studies of  $5\alpha$ -reductase type 2 deficiency provide additional evidence that T mediates the differentiation of Wolffian ducts. In the female, the Müllerian ducts give rise to the fallopian tubes, uterus and upper vagina, while the Wolffian ducts disappear or persist as a vestigial form as Gartner's ducts (Grumbach and Conte, 1999; Wilson *et al.*, 1993).

On the other hand, the external genitalia and the lower urogenital tracts of both sexes develop from common precursors, namely the genital tubercle, genital folds and genital swellings. The induction of male differentiation of the external genitalia and urogenitalia sinus is affected primarily by DHT. T is delivered by the bloodstream to these target tissues, that are rich in  $5\alpha$ -reductase and can convert T to DHT efficiently, even before the fetal testis

acquires the capacity to secrete T, which occurs at about week eight of human development. Complete external masculinization occurs only if the androgenic stimulus is received during the critical period ranging between 8 to 12 weeks of fetal life (Grumbach and Conte, 1999; Wilson *et al.*, 1993). Under the influence of DHT action, the genital tubercle elongates to form the body of the penis, and the urethral folds fuse ventrally from behind forward, to form the penile urethra. The labioscrotal swellings or folds grow toward each other, fusing in the midline to form the scrotum. DHT also induces the urogenital sinus to differentiate as the prostate, and inhibits the formation of the vesicovaginal septum, thereby preventing the development of the vagina. These processes are completed by week 12 of development (Quigley, 1998).

As in the case of the genital ducts, there is an inherent tendency for the external genitalia and urogenital sinus to feminize in the absence of fetal gonadal secretions. Indeed, the female phenotype does not require hormones from the fetal ovary. In the absence of DHT action, such as in the normal female fetus, the genital tubercle elongates only slightly to form the clitoris, the genital swellings become the labia majora and the genital folds become the labia minora. It is of interest to note that after approximately the 12th week, when the urogenital sinus has separated from the vagina, fusion of the labioscrotal folds and urethral groove cannot occur, even with an intense androgenic stimulus, whereas such a stimulation can cause clitoral hypertrophy at any time during fetal life and after birth (Grumbach and Conte, 1999).

### 17 $\beta$ -HYDROXYSTEROID DEHYDROGENASE DEFICIENCY

Since the 1950s, 17 $\beta$ -hydroxysteroid dehydrogenase/17-ketoreductase (17 $\beta$ -HSD/ KSR) activities have been characterized and the related enzymes have been purified from a large number of tissues from several species (Peltoketo *et al.*, 1999; Labrie *et al.*, 2000). 17 $\beta$ -HSD/KSR enzymes catalyze the interconversion of  $\Delta^4$ -DIONE into T, DHEA into  $\Delta^5$ -DIOL and E<sub>1</sub> into E<sub>2</sub>. Although eight enzymes, termed 17 $\beta$ -HSD/KSR types 1 to 8, which share low sequence similarity, have so far been cloned in mammals, these activities are mediated by seven known enzymes in the human (Peltoketo *et al.*, 1999). These enzymes are expressed individually in a cell-specific manner and have individual substrate specificity and distinct regulatory mechanisms, consequently providing each cell type with the means to precisely control the intracellular availability of each sex steroid according to local requirements (Labrie *et al.*, 1999). 17 $\beta$ -HSD/KSR deficiency is an autosomal recessive form of male pseudohermaphroditism, caused by impairment in the testicular conversion of  $\Delta^4$ -DIONE to T, resulting from mutations in the HSD17B3 gene encoding type 3 17 $\beta$ -HSD/ KSR. In order to better understand the consequences of a defect in 17 $\beta$ -HSD/KSR 3 activity, it is important firstly to provide a brief description of the key characteristics of the members of the human 17 $\beta$ -HSD/KSR family (Figure 11.1).

#### The human 17 $\beta$ -HSD/17-KSR gene family

The human type I 17 $\beta$ -HSD/KSR enzyme, the first 17 $\beta$ -HSD/KSR enzyme to be cloned, is a cytosolic protein that exists in a homodimeric form and catalyzes almost exclusively the

interconversion of E1 and E2 (Peltoketo *et al.*, 1988, 1992; The *et al.*, 1989; Luu-The *et al.*, 1990). This enzyme is a member of the short-chain alcohol dehydrogenase superfamily. The HSD17B1 gene, which is located on chromosome 17q21, in close proximity to the BRCA1 locus (Rommens *et al.*, 1995), is contained within a genomic fragment of 3.3kb and consists of six exons encoding a protein of 327 amino acids. It is also worthy of mentioning that human HSD17B1 is situated in tandem with a pseudogene that has been named HSD17BP1. Among mammalian steroidogenic enzymes, the crystallographic structure of type I 17 $\beta$ -HSD/KSR was the first to be determined (Azzi *et al.*, 1996; Ghosh *et al.*, 1995). This cytosolic type 1 enzyme uses NADP<sup>+</sup> (H) as the preferred cofactor, and is expressed at high levels in the ovary and placenta, where it primarily interconverts E<sub>1</sub> and E<sub>2</sub> (Luu-The *et al.*, 1989, 1990; Peltoketo *et al.*, 1988, 1992). Moreover, the expression of this isoenzyme has also been demonstrated in ZR-75-1 cells as well as in several other breast cancer cell lines and in human breast carcinoma (Martel *et al.*, 1992; Miettinen *et al.*, 1996; Sasano *et al.*, 1996).

The second 17 $\beta$ -HSD/KSR enzyme to be cloned, chronologically designated type 2 17 $\beta$ -HSD/KSR is abundantly expressed in both adult and fetal tissues including the placenta, uterus, liver and the gastrointestinal and urinary tracts (Casey *et al.*, 1994; Moghrabi *et al.*, 1997; Mustonen *et al.*, 1997; Mustonen *et al.*, 1998). The gene encoding type 2 17 $\beta$ -HSD/KSR (Labrie *et al.*, 1995) is located on chromosome 16q24 (Durocher *et al.*, 1995). The type 2 17 $\beta$ -HSD/KSR is a NAD<sup>+</sup>(H)-dependent membrane-associated protein consisting of 387 amino acids, which catalyzes, in cell homogenates, the interconversion of E<sub>2</sub> into E<sub>1</sub>, T into  $\Delta^4$ -DIONE and DHEA into  $\Delta^5$ -DIOL, and DHT into  $\Delta^4$ -DIONE. Nevertheless, because there is a much higher intracellular bioavailability of NAD<sup>+</sup> compared to NADH, this enzyme is especially responsible for the oxidative pathway, i.e., it is involved in the degradation of active estrogens and androgens. Consequently, due to the pattern of expression and enzyme characteristics of this isoenzyme, it has been suggested that the type 2 17 $\beta$ -HSD/KSR protects tissues from the actions of excess androgens and estrogens (Peltoketo *et al.*, 1999). Type 2 17 $\beta$ -HSD/KSR is also a member of the short-chain alcohol dehydrogenase super-family, but it shares only about 20% sequence identity with the cytoplasmic enzyme encoded by the HSD17B1 gene.

A third gene encodes a microsomal 17 $\beta$ -HSD/KSR protein comprising 310 amino acids, chronologically designated type 3, which uses NADP (H) as a cofactor (Geissler *et al.*, 1994). In contrast to the type 2 isoenzyme, due to the higher intracellular bioavailability of NADPH compared to NADP<sup>+</sup> within the cells expressing type 3 17 $\beta$ -HSD/KSR, this enzyme is responsible for the reductive pathway. The HSD17B3 gene was first located on chromosome 9q22 using somatic cell hybrid DNA panels and FISH analyses, thereafter, its mapping has been further localized *in silico* by our group close to marker D9S1717 using the Unified database for Human genome mapping (Figure 11.2). Microsomal type 3 17 $\beta$ -HSD/KSR shares 23% homology with the genes for type 1 and 2 17 $\beta$ -HSD/KSRs, and is almost exclusively expressed in the testis, where it is essential for T biosynthesis (Geissler *et al.*, 1994).

The type 4 17 $\beta$ -HSD/KSR is a unique multi-functional enzyme consisting of 17 $\beta$ -HSD/KSR-, hydratase- and sterol carrier 2-like domains and is ubiquitously expressed, although in certain tissues cell-specific expression has been demonstrated (Adamski *et al.*, 1992;

**Table 11.1** Genotype-phenotype relationships of patients with type 3 17 $\beta$ -HSD/KSR deficiency bearing mutation in the HSD17B3 gene. The ethnic origin, the phenotypic characteristics, reasons for referral and clinical features of the patients are indicated as well as a summary of the functional consequence of mutations in the HSD17B3 gene.

Family	Origin	Karyotype	Patient	Age at diagnosis	Phenotype or reason for referral	Consanguinity	Case report and Mutation report	Mutant alleles	Apparent activity
<i>Homozygotes</i>									
1	Pakistani	46XY	1	12.3 years	Virilization, clitoromegaly, palpable inguinal gonads	Yes	(Moghrabi <i>et al.</i> , 1998)	A56T	Yes
2	Iranian	46XY	2	16 years	Hirsutism, amenorrhea	N/A	(Andersson <i>et al.</i> , 1996)	S65L	No
3	Algerian	46XY	3	13.5 years	clitoromegaly	Yes	(David <i>et al.</i> , 1975; Calemard-Michel <i>et al.</i> , 1996a)	N74T	?
4	Dutch	46XY	4	4 years	Abnormal genitalia	No	(Boehmer <i>et al.</i> , 1999)	N74T	?
5	Dutch	46XY	5	16 years	Virilization at puberty	Yes	(Boehmer <i>et al.</i> , 1999)	N74T	?
6	Turkish	46XY	6	11 months	Moderate clitoromegaly post labia fusion, palpable gonads	Yes	(Calemard-Michel <i>et al.</i> , 1996c)	R80Q	Yes
7	Dutch	46XY	7	13.5 years	Virilization, clitoromegaly, post labia fusion	No	(Boehmer <i>et al.</i> , 1999)	R80Q	Yes
8	Large Arabian kinship from Gaza strip	46XY	8	13 years	Virilization at puberty	Yes	(Kohn <i>et al.</i> , 1985; Geissler <i>et al.</i> , 1994)	R80Q	Yes
9	Arabian kinship from Gaza strip (9 families)	46XY	10-30		Males have genital ambiguity	Yes	(Geissler <i>et al.</i> , 1994; Rosler <i>et al.</i> , 1996)	R80Q	Yes
		46XX	31-33		Females are asymptomatic				
10	Brazilian	46XY	34	4 years	Clitoromegaly, post labia fusion, palpable inguinal mass	No	(Arnhold <i>et al.</i> , 1988; Andersson <i>et al.</i> , 1996)	R80Q	Yes



Family	Origin	Karyotype	Patient	Age at diagnosis	Phenotype or reason for referral	Consanguinity	Case report and Mutation report	Mutant alleles	Apparent activity
		46XY	35	10 years	Presumptive testicular feminization, family history		(Arnhold <i>et al.</i> , 1988; Andersson <i>et al.</i> , 1996)		
		46XX	36	11 years	Normal (Asymptomatic)		(Geissler <i>et al.</i> , 1994; Mendonca <i>et al.</i> , 1999)		
11	Spanish	46XY	37	4.5 months	Female external genitalia with gonadal masses in labia majora	No	(Bilbao <i>et al.</i> , 1998)	R80W	?
12	French	46XY	38	7 weeks	Gonadal hernia, moderate clitoromegaly	No	(David <i>et al.</i> , 1997)	G178D	?
13	French	46XY	39	17 years	Amenorrhea, clitoromegaly, gonads in labia majora	No	(Tourniaire, 1973; Calenard-Michel <i>et al.</i> , 1996a)	S184F	?
		46XY	40	18 years	Amenorrhea, clitoromegaly, gonads in labia majora				
		46XY	41	15 years	Amenorrhea, clitoromegaly, gonads in labia majora				
14	Turkish	46XY	42	2 years	Inguinal mass	No	(Boehmer <i>et al.</i> , 1999)	A188V	?
15	African-Brazilian	46XY	43	13 years	Amenorrhea, hirsutism, clitoromegaly, gonads in inguinal canals	Yes	(Geissler <i>et al.</i> , 1994)	A203V	No
16	German	46XY	44	Birth	Clitoromegaly, posterior labia fusion	Yes	(Andersson <i>et al.</i> , 1996)	F208I	No
17	Brazilian	46XY	45	21 years	Raised as a girl until age 10. Bifid scrotum, inguinal mass	Yes	(Andersson <i>et al.</i> , 1996)	E215D	No
		46XX	46	11 years	Normal (Asymptomatic)		(Andersson <i>et al.</i> , 1996; Mendonca <i>et al.</i> , 1999)		

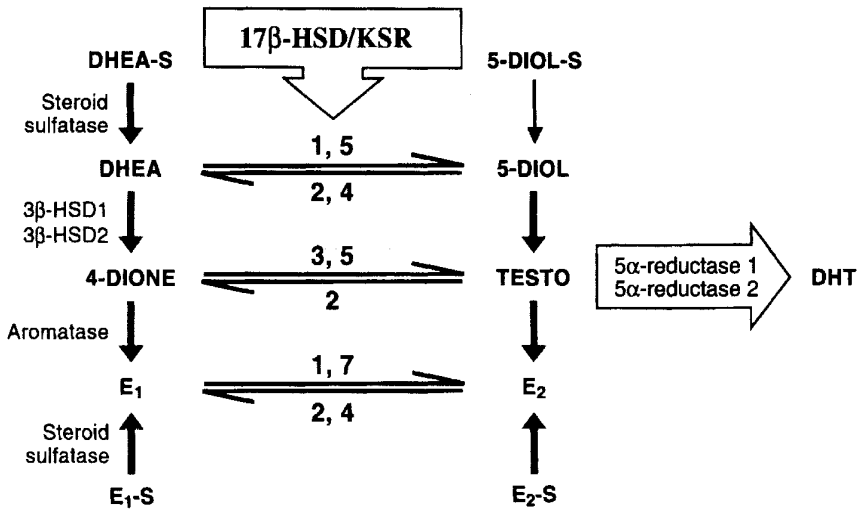
	46XX	47	11 years	Normal (Asymptomatic)	(Andersson <i>et al.</i> , 1996; Mendonca <i>et al.</i> , 1999)	
18	Dutch	48	0.8 years	Inguinal mass	(Boehmer <i>et al.</i> , 1999)	325 + 4; A → T
19	Dutch	49	0.3 years	Family history		No predicted activity
	46XX	50	0.1 years	Inguinal mass	(Boehmer <i>et al.</i> , 1999)	325 + 4; A → T
20	Dutch	51	Birth	Inguinal mass	(Andersson <i>et al.</i> , 1996; Boehmer <i>et al.</i> , 1999)	325 + 4; A → T
	46XX	51	Birth	Inguinal mass		No predicted activity
21	Dutch	52	Birth	Abnormal genitalia	(Boehmer <i>et al.</i> , 1999)	325 + 4; A → T
22	American	53	15 years	Clitoromegaly, scrotalization of labia majora, gynaecomastia, virilization, blind-ending vagina	(Andersson <i>et al.</i> , 1996)	325 + 4; A → T
23	American	54	13 years	Clitoromegaly, virilization, inguinal masses, blind-ending vagina	(Andersson <i>et al.</i> , 1996)	325 + 4; A → T
24	German	55	3 weeks and 0.5 years	Testicular masses in the labia majora	(Andersson <i>et al.</i> , 1996)	325 + 4; A → T
25	Dutch	56	8 years	Inguinal mass	(Boehmer <i>et al.</i> , 1999)	326 - 1; G → C
26	Brazilian	57	34 years	Hirsutism, gonads palpable in inguinal canals	(Geissler <i>et al.</i> , 1994)	326 - 1; G → C
	46XX	58	35 years	Normal (Asymptomatic)	(Geissler <i>et al.</i> , 1994; Mendonca <i>et al.</i> , 1999)	No predicted activity
	46XX	59	28 years	Normal (Asymptomatic)	(Geissler <i>et al.</i> , 1994; Mendonca <i>et al.</i> , 1999)	No predicted activity

Family Origin	Karyotype	Patient Age at diagnosis referral	Phenotype or reason for referral	Consanguinity	Case report and Mutation report	Mutant alleles	Apparent activity
27 Syrian	46XY	60	17 years Gynaecomastia	No	(Ademola Akesode <i>et al.</i> , 1977; Geissler <i>et al.</i> , 1994)	655 - 1; G → A	No predicted activity
28 Greek	46XY	61	12 years Clitoromegaly, presence of testes	No	(Virdis <i>et al.</i> , 1978; Geissler <i>et al.</i> , 1994)	655 - 1; G → A	No predicted activity
29 Turkish	46XY	61	18 years Pseudovaginal perineoscrotal hypospadias, testes in bifid scrotum, female escutcheon	Yes	(Imperato-McGinley <i>et al.</i> , 1987; Can <i>et al.</i> , 1998)	655 - 1; G → A	No predicted activity
	46XY	62	45 years Ambiguous genitalia, moderate amount of facial hair		(Can <i>et al.</i> , 1998)		
30 Turkish	46XY	63	16 years Virilization at puberty	Yes	(Boehmer <i>et al.</i> , 1999)	655 - 1; G → A	Variable (small amount of wild-type mRNA)
	46XY	64	15 years Family history				No predicted activity
	46XY	65	10 years Family history				
31 Algerian	46XY	66	3 years Palpable gonads in pseudoscrotal labia majora	Yes	(Forest <i>et al.</i> , 1989; Calemard-Michel <i>et al.</i> , 1996c)	702 insAA	No predicted activity
	46XY	67	1 weeks Gonadal hernia		(Calemard-Michel <i>et al.</i> , 1996c; Forest <i>et al.</i> , 1989)		
	46XX	68	17 years Asymptomatic		(Calemard-Michel <i>et al.</i> , 1999)		
32 Polish	46XY	69	10, 13 and 15 years Testis in right herniorrhaphy sac, gynaecomastia, amenorrhoea	No	(Andersson <i>et al.</i> , 1996)	del777 → 783	No predicted activity

*Compound heterozygotes*

33	African-American	46XY	70	17 years	Clitoromegaly and failure to menstruate	No	(Geissler <i>et al.</i> , 1994)	S232L/M235V	No/No
34	American	46XY	71	14 years	Testes in inguinal canals	N/A	(Moghrabi <i>et al.</i> , 1998)	N130S/G289S	Yes/Yes (Neutral polymorphism)
35	West Indian	46XY	72	Birth	Abnormal genitalia	No	(Wit <i>et al.</i> , 1988), (Boehmer <i>et al.</i> , 1999)	N130S/G289S	Yes/Yes (Neutral polymorphism)
36	Dutch	46XY	73	14 years	Virilization at puberty	No	(Boehmer <i>et al.</i> , 1999)	N74T/325 + 4; A → T	?/No predicted activity
37	Dutch	46XY	74 75	10 years 0.1 years	Family history Inguinal mass	No	(Boehmer <i>et al.</i> , 1999)	N74T/325 + 4; A → T	?/No predicted activity
38	Dutch	46XY	76	0.1 years	Inguinal mass	No	(Boehmer <i>et al.</i> , 1999)	R80Q/325 + 4; A → T	Yes/No predicted activity
39	Dutch	46XY	77	Birth	Abnormal genitalia	No	(Boehmer <i>et al.</i> , 1999)	R80Q/325 + 4; A → T	Yes/No predicted activity
40	Italian German-Irish	46XY	78	13 years	Clitoromegaly, scrotalization of labia majora, inguinal masses, blind-ending vagina	No	(Andersson <i>et al.</i> , 1996; Moghrabi <i>et al.</i> , 1998)	Q176P/325 + 4; A → T	Yes/No predicted activity
41	American	46XY	79	13 years	Clitoromegaly, inguinal mass, blind-ending vagina	No	(Andersson <i>et al.</i> , 1996)	P282L/325 + 4; A → T	No/No predicted activity
42	Dutch	46XY	80 81	12 years 2 years	Clitoromegaly, testis-like masses in inguinal region	No	(Boehmer <i>et al.</i> , 1999)	325 + 4; A → T/ 326 - 1; G → C	No predicted activity
43	Dutch	46XY	82	0.1 years	Inguinal mass	No	(Boehmer <i>et al.</i> , 1999)	325 + 4; A → T/ 326 - 1; G → C	No predicted activity

Family	Origin	Karyotype	Patient	Age at diagnosis	Phenotype or reason for referral	Consanguinity	Case report and Mutation report	Mutant alleles	Apparent activity
44	Brazilian	46XY	83	15 years	Inguinal mass	No	(Geissler <i>et al.</i> , 1994)	R80Q/326 - 1; G → C	Yes/No predicted activity
		46XX	84	50 years	Normal (Asymptomatic)		(Geissler <i>et al.</i> , 1994; Mendonca <i>et al.</i> , 1999)		
		46XX	85	12 years	Normal (Asymptomatic)		(Mendonca <i>et al.</i> , 1999)		
45	French	46XY	86	14 months	Bilateral gonadal hernia, labia fusion, palpable gonads	No	(Calenard-Michel <i>et al.</i> , 1996b)	325 + 4; A → T/ 655 - 2; A → G	No predicted activity
46	American	46XY	87	Birth	Perineoscrotal hypos-padias, bifid scrotum	No	(Andersson <i>et al.</i> , 1996)	V205E/?	No

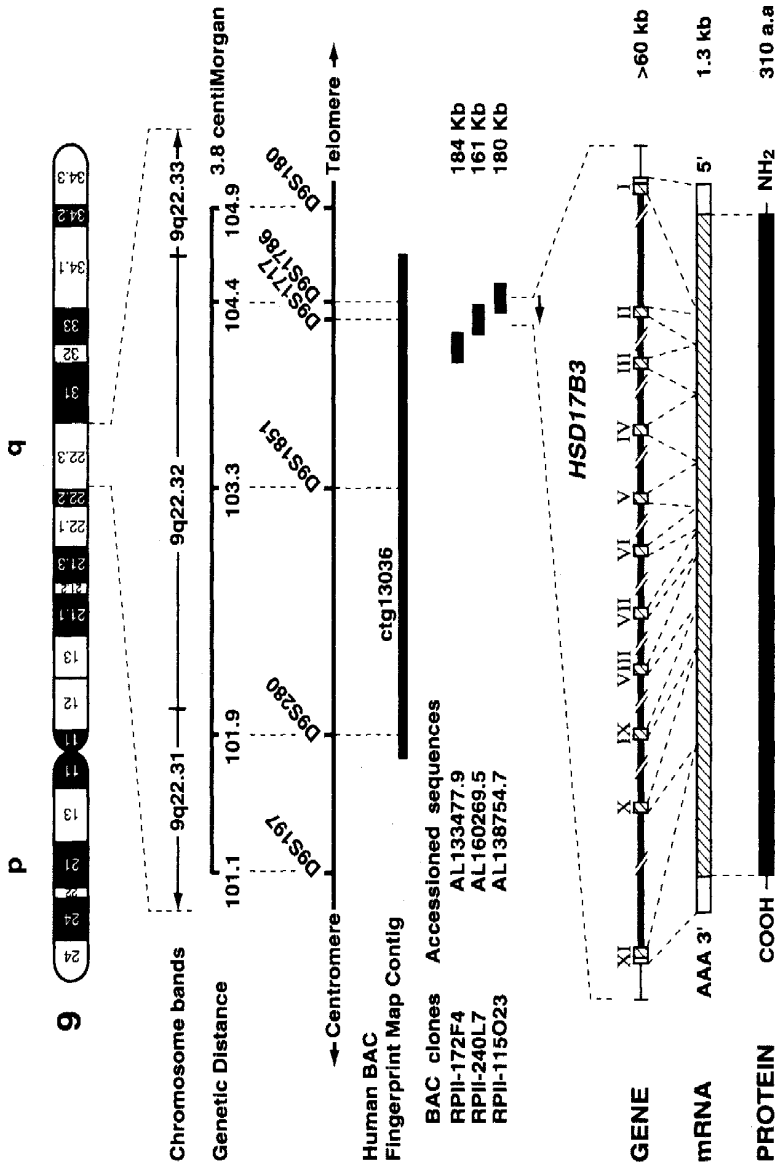


**Figure 11.1 Main biosynthetic and inactivating pathways of androgens and estrogens in the human.** The various types of 17 $\beta$ -HSDs are indicated as numbers as well as the direction of their enzymatic activity.

Leenders *et al.*, 1996; Qin *et al.*, 1997). This peroxisomal enzyme is a NAD<sup>+</sup>(H)-dependent 736-amino acid enzyme, which is cleaved to form a 32kDa N-terminal fragment. Both the full length (80kDa) and the 32kDa peptide catalyze the D-specific dehydrogenase reaction of 3-hydroxyacyl-CoA, E<sub>2</sub> and  $\Delta^5$ -DIOL. Mutations in the HSD17B4 gene located on 5q2.3 lead to a peroxisomal D-hydroxy-acyl-coA dehydrogenase deficiency known as Zellweger syndrome (Novikov *et al.*, 1997; van Grunsven *et al.*, 1998).

While type 3 17 $\beta$ -HSD/KSR synthesizes T from  $\Delta^4$ -DIONE in the Leydig cells of the testis, the same enzymatic reaction is catalyzed in peripheral target tissues by a different enzyme, namely type 5 17 $\beta$ -HSD/KSR. The latter enzyme is also known as type 2 3 $\alpha$ -HSD or AKR1C3, and in contrast to other 17 $\beta$ -HSD/KSRs it belongs to the aldoketoreductase protein family (Peltoketo *et al.*, 1999). This cytosolic NADP (H)-dependent enzyme is encoded by a gene located on chromosome 10p 14–15 and shares a high sequence identity with members of the 3 $\alpha$ -HSD family, although it possesses a strong ketoreductase activity (Deyashiki *et al.*, 1995; Khanna *et al.*, 1995; Lin *et al.*, 1997; Dufort *et al.*, 1999). The enzyme is expressed in several tissues including the liver, placenta, adrenal, testis, the basal cells of the prostate and in prostatic carcinoma cell lines (Lin *et al.*, 1997; Dufort *et al.*, 1999; El-Alfy *et al.*, 1999).

Using a rat prostate cDNA library, obtained by expression cloning, Biswas and Russell (Biswas and Russell, 1997) isolated several cDNA clones which were demonstrated to metabolize 3 $\alpha$ -diol. Among the many clones obtained, one, termed type 6 17 $\beta$ -HSD/KSR, selectively catalyzes the oxidation of 3 $\alpha$ -diol into androsterone. The transformation of other C<sub>19</sub>-steroids, namely DHT to androstanedione and T to  $\Delta^4$ -DIONE also occurs but at rate approximately 50- to 100-folds lower. The type 6 17 $\beta$ -HSD/KSR shares 65% sequence



**Figure 11.2 Fine chromosomal localization of the HSD17B3 gene.** Location in centiMorgan of each marker was determined according to the genetic linkage map of genethon. The human BAC fingerprint map contig was obtained by using the Human Genome Project Working Draft of the Washington University Genome Sequencing Center, St. Louis, MO, USA (<http://genome.ucsc.edu/goldenPath/hgTracks.html>). The clones come from the Pieter J. de Jong RPc1-11 libraries (Children's Hospital Oakland—BACPAC Resources Oakland, CA, USA).

identity with rat type 1 retinol dehydrogenase (RoDHL), therefore belonging to the retinol dehydrogenase family. The human counterpart for this isoenzyme has not yet been described.

The gene for type 7 17 $\beta$ -HSD/KSR was assigned to human chromosome 10p 11.2 and encodes a protein of 341 amino acid residues (Krazeisen *et al.*, 1999). This type of 17 $\beta$ -HSD/KSR has been first characterized in rodents (Peltoketo *et al.*, 1999). Both rat and mouse type 7 17 $\beta$ -HSD/KSR catalyze exclusively the conversion of E<sub>1</sub> to E<sub>2</sub> and is also abundantly expressed especially in the corpus luteum during the second half of rodent pregnancy (Nokelainen *et al.*, 1998). In human, this NADP(H)-dependent enzyme is also present in corpus luteum, placenta and several other steroid target-tissues including the breast, testis, prostate and liver (Krazeisen *et al.*, 1999).

Finally, 17 $\beta$ -HSD/KSR type 8 is encoded by the HKE6 gene, which is located in the HLA region on chromosome 6p21–3 (Kikuti *et al.*, 1997). This gene product has been characterized as a protein whose abnormal regulation is linked to the development of recessive polycystic kidney disease in two different models of PKD mice (Azziz *et al.*, 1993). Nevertheless, further analysis would be required to provide a direct evidence of the involvement in this particular disorder. This enzyme is believed to inactivate E<sub>2</sub> to E<sub>1</sub> and, to a certain extent, the oxidation of androgens (Fomitcheva *et al.*, 1998).

### Clinical features of 17 $\beta$ -HSD/KSR deficiency

During the period from 1965–1970 there was a certain amount of confusion concerning the correct terminology to use for this particular disease. In certain cases, individuals were labeled as suffering from the incomplete form of Morris syndrome, due to the presence of gynecomastia. Indeed, in 1966, Neher and Kahnt (Neher and Kahnt, 1965) first reported the anomaly involved in the biosynthesis of estrogens/androgens in a patient identified to be suffering from Morris syndrome. The correct diagnosis of 17 $\beta$ -HSD/KSR deficiency was not assigned in this patient until a few years later (Zurbrugg, 1974). Thereafter, several cases of 17 $\beta$ -HSD/KSR deficiency were described in individuals who did not present with gynecomastia (Givens *et al.*, 1974; Forest *et al.*, 1979), while some of these individuals were described with pseudovaginal perineoscrotal hypospadias (Opitz *et al.*, 1972) or were assigned to have androgen insensitivity syndrome (Knorr *et al.*, 1973).

Male pseudohermaphroditism caused by 17 $\beta$ -HSD/KSR deficiency, was first reported by Saez and colleagues (Peretti *et al.*, 1970; Saez *et al.*, 1971; Saez *et al.*, 1972). Their first description of this deficiency resulted from an *in vitro* (Saez *et al.*, 1971) and an *in vivo* (Saez *et al.*, 1972) study in a patient, who was raised as a girl, but at puberty presented with signs of intense virilization and gynecomastia. Following this initial characterization of 17 $\beta$ -HSD/KSR deficiency, many patients were then identified to be suffering from this disease, including a cohort of 68 subjects from an highly inbred population in the Gaza Strip (Table 11.1) (Forest *et al.*, 1989; Forest, 1995; Grumbach and Conte, 1999).

Most 46XY individuals with deficiency in testicular type 3 17 $\beta$ -HSD/KSR activity have external female genitalia or a slightly enlarged clitoris, although their internal genitalia are well masculinized, with normally formed epididymis, vas deferens, seminal vesicles and blind vaginal pouch. The relatively normal masculinization of internal structures may result from



the activity of another  $17\beta$ -HSD/ KSR isoenzyme (s) in the proximity of the Wolffian ducts which ensures adequate local production of T from testis-derived  $\Delta^4$ -DIONE during the appropriate period of fetal life. However, at puberty, gonadotropin levels and plasma concentrations of  $\Delta^4$ -DIONE,  $E_1$  and T increase, therefore breast enlargement is observed at puberty in half of the patients, which most likely results from the conversion of  $\Delta^4$ -DIONE to  $E_1$  by aromatase in peripheral tissues, followed by its conversion into  $E_2$  via the action of other wild-type  $17\beta$ -HSD/KSR(s). On the other hand, considerable virilization of the external genitalia occurs at the time of puberty which is associated, in the majority of affected individuals, with the pattern of male hair growth, deepening of the voice and increased muscle mass (Rosler *et al.*, 1992; Andersson *et al.*, 1996; Grumbach and Conte, 1999). This apparent "recovery" in  $17\beta$ -HSD/KSR activity, which takes place at the time of puberty, results from the conversion of  $\Delta^4$ -DIONE to T via the action of type 5  $17\beta$ -HSD/ KSR in peripheral tissues, which is consistent with the initial findings showing that peripheral  $17\beta$ -HSD/KSR activity is normal, as evidenced by studies monitoring the fate of injected labeled T (Saez *et al.*, 1971), or *in vitro* studies in skin (Wilson *et al.*, 1987).

Polycystic ovarian disease in women has been associated with elevated plasma  $\Delta^4$ -DIONE levels, which was thought to result from type 3  $17\beta$ -HSD/KSR deficiency (Pang *et al.*, 1987; Toscano *et al.*, 1990), but subsequently it has been shown that women with mutations in the HSD17B3 gene were asymptomatic (Wilson *et al.*, 1987; Rosler *et al.*, 1996; Mendonca *et al.*, 1999). Therefore it is likely that another  $17\beta$ -HSD/KSR enzyme is responsible for this apparent enzyme deficiency, particularly considering that the type 3 is not significantly expressed in the ovary (Zhang *et al.*, 1996), in comparison to the type 5, which is expressed (Luu-The *et al.*, 2001; Pelletier *et al.*, 1999).

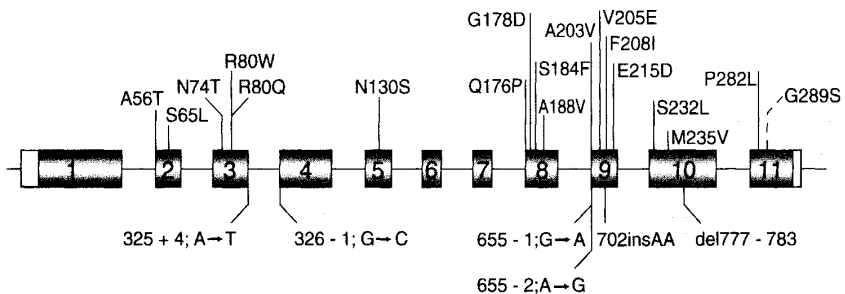
### Biological diagnosis

The diagnosis of type 3  $17\beta$ -HSD/ KSR deficiency is made when a 10- to 15-fold elevation in the ratios of  $\Delta^4$ -DIONE to T is observed. The accumulation of  $\Delta^4$ -DIONE in the circulation while the plasma levels of T is low or near normal is observed in the perinatal period, following hCG stimulation in childhood, or after puberty in male pseudohermaphrodites who have no abnormality in adrenal steroid biosynthesis and who undergo virilization at puberty, either with or without gynecomastia (Forest, 1995). The ratio of  $\Delta^4$ -DIONE/T can be used diagnostically, even in the basal state, in pubertal individuals or adult patients diagnosed with  $17\beta$ -HSD/ KSR deficiency, since the hyperplastic Leydig cells predominantly secrete  $\Delta^4$ -DIONE. Moreover, Forest has also stated that when there is insufficient gonadotropic stimulation, the prepubertal testis does not produce excessive amounts of  $\Delta^4$ -DIONE, therefore, in contrast to pubertal subjects, baseline levels of  $\Delta^4$ -DIONE are normal in pre-pubertal patients and the diagnosis can only be made when the ratio of  $\Delta^4$ -DIONE/T after hCG stimulation is high ( $>1$ ) (Forest, 1995). The testicular origin of the  $\Delta^4$ -DIONE overproduction is consistent with the finding that high  $\Delta^4$ -DIONE levels are not suppressed by dexamethasone, but are reduced by exogenous administration of androgens or estrogens (Forest *et al.*, 1989; Forest, 1995). Moreover, plasma levels of FSH are normal or subnormal while those of LH are constantly elevated. The reduced activity of testicular type 3  $17\beta$ -

HSD/KSR is also inferred by the finding of elevated ratios of  $\Delta^4$ -DIONE to T and E<sub>1</sub> to E<sub>2</sub> in spermatic venous samples.

### Molecular diagnosis

Several mutations have been identified in the HSD17B3 gene in more than 87 individuals suffering from 17 $\beta$ -HSD/KSR deficiency originating from approximately 46 distinct families (Table 11.1). Eleven females (R80Q/R80Q: patients 31–33 and 36; E215D/E215D: patients 46 and 47; 326–1; G→C/326–1; G→C: patients 58 and 59; 702 insA: patient 68; R80Q/326–1; G→C: patients 83 and 84) have been identified to carry mutations, but they were all asymptomatic (Rosler *et al.*, 1996; Mendonca *et al.*, 1999). The mutations identified to date include 18 single-base mutations resulting in amino acid substitutions, two frameshift mutations leading to truncated proteins and four splice junction mutations causing abnormal mRNA splicing, which results in disturbed protein structure and enzyme function (Figure 11.3). At least the four recurrent mutations, 325+4; A→T, 655–1; G→C, R80Q and N74T, found worldwide appear to be ancient and appear to originate from genetic founders (Boehmer *et al.*, 1999). On the other hand, these authors have also provided evidence suggesting that the recurrent mutations 326–1; G→C and P282L were *de novo* mutations. An interesting founder effect is represented by the R80Q mutation which is common among Arabs in various parts of Israel (from Gaza, Jerusalem, and Lod-Ramle) with some individuals having Druze ancestry from Syria and Lebanon (Rosler *et al.*, 1996). It has recently been speculated that the detection of this mutation in Portuguese, Spanish, Dutch and white Brazilians is consistent with the hypothesis that the R80Q mutation was introduced by the Phoenicians, who migrated from an area in present day in Syria, Lebanon and Israel around 750 BC towards Portugal and Spain (Boehmer *et al.*, 1999).



**Figure 11.3 Representation of all the mutations identified to date in the HSD17B3 gene in individuals suffering from type 3 17 $\beta$ -HSD/17-KSR deficiency.** All the missense mutations are shown in the upper panel, while the frameshift and splice-junction mutations are shown below. The location of mutation G289S in the HSD17B3 gene is represented by a broken line since this mutation is supposed to be a neutral polymorphism (Moghrabi *et al.*, 1998).

It is also of interest to note that as in the case of other autosomal recessive diseases, type 3 17 $\beta$ -HSD/KSR deficiency may show increased frequencies among populations with a high

intermarriage rate. Indeed in Arabs in Gaza, the incidence of homozygotes for the R80Q mutation is approximately 1/200 to 1/300. In contrast, frequency for this disease resulting from multiple mutations in the HSD17B3 gene in the Caucasian Dutch population in the Netherlands, is estimated to a minimal incidence of the disease of 1/147000 (Boehmer *et al.*, 1999).

Among the 12 missense mutations that have been functionally characterized and enzymatic activity assessed, only four mutations were found to retain residual type 3  $17\beta$ -HSD/KSR activity, while the neutral polymorphic G289S substitution, which has a frequency of 0.06, resulted in an enzyme that possessed kinetic properties similar to those of the wild type enzyme (Moghrabi *et al.*, 1998). In brief, mutation R80Q appears to possess an apparent residual amount of enzyme activity, i.e., approximately 20% of the activity of the wild-type enzyme, with a  $V_{\max}$  of 14 pmol min<sup>-1</sup> mg<sup>-1</sup> cell lysate protein, in contrast to the  $V_{\max}$  of the wild-type which is 99 pmol min<sup>-1</sup> mg<sup>-1</sup> (Geissler *et al.*, 1994). Furthermore, mutations A56T, N130S and Q176P were also found to severely compromise enzyme activity. For example the A56T mutation caused a 6-fold increase in the apparent  $K_m$  value for  $\Delta^4$ -DIONE, while a 20-fold increase in the apparent  $K_m$  value for the cofactor, NADPH, was also observed. It is of interest to note that this mutation disturbed the dinucleotide binding domain which is located in the amino-terminal region in all members of the short-chain alcohol dehydrogenase superfamily with the characteristic G-X-X-X-G-X-G motif (Scrutton *et al.*, 1990). This is reminiscent of the G15D mutation in the HSD3B2 gene which was also reported to decrease the affinities for both the cofactor and the substrate (Rheume *et al.*, 1995). Although N130S and Q176P mutant proteins have low activity in transfected intact cells, accurate kinetic constants using cell lysates could not be determined for mutations N130S and Q176P (Moghrabi *et al.*, 1998), even when the protein stabilizing agent glycerol was used in assays under conditions previously proven to be successful for reconstituting the unstable A245P type II  $3\beta$ -HSD mutant protein (Simard *et al.*, 1993). However, no significant activity could be detected for missense mutations S65L, A203V, V205E, F208I, E215D, S232L, M235V, P282L or deletion  $\Delta 777-783$  (Geissler *et al.*, 1994; Andersson *et al.*, 1996), while more recently reported mutations N74T, A188V (Boehmer *et al.*, 1999), R80W (Bilbao *et al.*, 1998), G178D (David *et al.*, 1997) and S184F (Calemard-Michel *et al.*, 1996) have not yet been characterized.

Because all the missense mutations located in exon 9 (Figure 11.3) that have been tested were found to completely abolish enzyme activity (Geissler *et al.*, 1994; Moghrabi *et al.*, 1998), the transcripts with a deletion of exon 9 or a deletion of both exon 9 and 10, identified in patient 65 bearing the homozygous 655-1; G→C mutation, are most likely to be nonfunctional (Boehmer *et al.*, 1999). Moreover, it is tempting to propose a similar conclusion for the heterozygous 655-2; A→G mutation found in patient 86 (Table 11.1). On the other hand, the presence of a transcript corresponding to the wild-type enzyme in testicular RNA from patient 63 bearing the homozygous 655-1; G→C mutation, suggests that phenotypic variation between homozygotes for this mutation could occur, depending on the amount of wild-type transcript formed (Boehmer *et al.*, 1999). Because the outcome of aberrant splicing is variable, the absence of a wild-type transcript in patients homozygous for 325+4; A→T or 326-1; G→C does not exclude the possibility of the presence of the wild-type transcript in other patients (Boehmer *et al.*, 1999). Consequently phenotypic variation

between families was reported for the same mutation (Table 11.1). In conclusion it seems clear that no specific phenotype is associated with a specific mutation in HSD17B3 in patients suffering from 17 $\beta$ -HSD/KSR deficiency (Boehmer *et al.*, 1999).

### Management

In general, due to the absence of ambiguous female genitalia in certain individuals at birth, these individuals are usually assigned as being female and raised as such, with the suggestion of utilizing pre-pubertal castration and estrogen replacement therapy (Forest, 1995), although with a certain amount of controversy (Grumbach and Conte, 1999). Taking advantage of the recent advances in molecular neonatal diagnostics, the use of T therapy in infancy to augment phallic size and genitoplasty have been suggested for patients with ambiguous genitalia who are to be raised as males (Grumbach and Conte, 1999). Such T replacement therapy is also necessary at the age of puberty in order to achieve complete masculinization and to prevent the appearance of gynecomastia (Grumbach and Conte, 1999). In this respect, like patients suffering from 5 $\alpha$ -reductase type 2 deficiency (Wilson, 1982; Wilson *et al.*, 1993), many affected individuals from a large Arab kindred from Gaza spontaneously adopt the male gender role, with apparently adequate sexual function, but who are infertile (Rosler and Kohn, 1983). However, the decision to adopt a male gender role may be prompted by the advantage in being a male in this particular society (Forest, 1995). It is important to mention that in both patients raised as males and in those individuals who change their gender to male at puberty and retain their testis, cryptorchidism and elevated gonadotropin levels post-pubertally may increase the risk of testicular malignancy (Forest, 1995; Grumbach and Conte, 1999). In fact, as discussed by Forest (Forest, 1995) the question of whether exposure to high  $\Delta^4$ -DIONE levels *in utero* and post-natally leads to disturbed female gender identity in later life remains largely unsettled. Consequently, the best management strategy for such individuals will be dictated by the age at diagnosis and the familial or social context.

### 5 $\alpha$ -REDUCTASE DEFICIENCY

The 5 $\alpha$ -reductase activity was initially characterized in the 1950s in rat liver slices based on its capacity to convert deoxycorticosterone to 5 $\alpha$ -reduced metabolites. Thereafter, Tomkins and others demonstrated that this enzyme used NADPH as cofactor and was able to catalyze the 5 $\alpha$ -reduction of a variety of steroid substrates (Russell and Wilson, 1994). The 5 $\alpha$ -reduction of steroid substrates renders their 3-oxo groups more susceptible to reduction by 3 $\alpha$ -HSDs and 3 $\beta$ -HSDs and to sulfation and glucuronidation. Because the latter modifications decrease the affinity of the steroid for its receptor, make it more hydrophilic, and facilitate its excretion, the 5 $\alpha$ -reductase was believed to be predominantly involved in the catabolism of steroids. The subsequent observation in the 1960s showing that 5 $\alpha$ -reductase is unable to catalyze the dehydrogenation of reduced steroids, led to the hypothesis that 5 $\alpha$ -reduction might be a regulatory step.

The notion that the 5 $\alpha$ -reduction of T is a crucial step in androgen action was further supported by the finding that DHT is a more potent androgen than T in the rat ventral

prostate and the observation that the administration of radiolabeled T to rats resulted in a time-dependent accumulation of DHT in the nuclei of responsive cells such as ventral prostate cells (Russell and Wilson, 1994). The key role of 5 $\alpha$ -reductase in male sexual differentiation was first recognized by the observation in mammalian embryos that this activity was highest in the primordia of the prostate and external genitalia prior to their virilization but very low in wolffian duct structures (Wilson and Lasnitzki, 1971; Wilson, 1972; Siiteri and Wilson, 1974). Thereafter, the formal genetic proof of the crucial role of 5 $\alpha$ -reductase in male sexual differentiation was provided by the demonstration that a rare autosomal recessive disorder, originally termed pseudovaginal perino-scrotal hypospadias (Nowakowski and Lenz, 1961), was caused by a defect of 5 $\alpha$ -reductase activity (Imperato-McGinley *et al.*, 1974; Walsh *et al.*, 1974).

The molecular features of 5 $\alpha$ -reductase deficiency were elucidated in fibroblasts cultured from the genital skin of affected individuals (Moore *et al.*, 1975; Wilson, 1975; Moore and Wilson, 1976). In contrast to normal fibroblasts, cells isolated from these patients failed to show 5 $\alpha$ -reductase activity with an acidic pH optimum. A second activity was detected at an alkaline pH optimum in both genital and non genital fibroblasts from affected individuals. Further insight into the meaning of these findings and the existence of multiple isoenzymes was hampered by the extreme insolubility of the protein. The characterization of cDNA clones obtained by using expression cloning strategy provided the molecular explanation for these two activities, which are in fact encoded by two different genes (Andersson and Russell, 1990; Andersson *et al.*, 1991; Jenkins *et al.*, 1992). The first enzyme cloned was designated steroid 5 $\alpha$ -reductase type 1 and exhibits an alkaline pH optimum, whereas the acidic pH enzyme was termed steroid 5 $\alpha$ -reductase type 2.

### The human 5 $\alpha$ -reductase gene family

#### *Structure and function*

The purification impasse was broken in 1989 when the expression cloning strategy in *Xenopus laevis* oocytes was successfully used to isolate a cDNA from rat liver encoding a 5 $\alpha$ -reductase isoenzyme. The human ortholog of the rat 5 $\alpha$ -reductase type 1 was then elucidated by cross-hybridization screening of a prostate cDNA library (Andersson and Russell, 1990). The 5 $\alpha$ -reductase type 1 has a broadly alkaline pH optima that spans from pH 6.0 to 8.5, has an apparent  $K_m$  in the micromolar range and was reported to be normal in patients suffering from 5 $\alpha$ -reductase deficiency (Jenkins *et al.*, 1992). The human enzyme is composed of 259 amino acids and is encoded by the SRD5A1 gene, which is located on the distal short arm of chromosome 5p15. The processed pseudogene SRD5AP1 apparently derived from a transcript of SRD5A1 gene maps on chromosome X q24-qter (Jenkins *et al.*, 1991).

The second 5 $\alpha$ -reductase was isolated by using expression cloning strategy in human cultured cells, possesses an acidic pH optimum, has an apparent  $K_m$  in the submicromolar range and is sensitive to finasteride, a powerful 4-azasteroid inhibitor of this activity in the prostate (Andersson *et al.*, 1991). The 5 $\alpha$ -reductase type 2 enzyme is composed of 254 amino acids and is encoded by the SRD5A2 gene located on chromosome 2p22.3 (Figure 11.4) (Morissette *et al.*, 1996). Both enzymes share approximately 47% identity and

they have similar gene structure with five exons each (Jenkins *et al.*, 1991; Labrie *et al.*, 1992; Thigpen *et al.*, 1992b). It is of interest to note that the *Arabidopsis* DET2 protein shares 38–42% sequence identity with mammalian 5 $\alpha$ -reductase isoenzymes and has a similar hydropathy profile. The structural and functional conservation between human 5 $\alpha$ -reductases and the DET2 gene of this plant raise interesting issues concerning the evolutionary origin of the steroid signaling system, which evolved before plants and animals diverged from protists (Li *et al.*, 1997).

#### *Tissue distribution and cell type specific expression*

Russell's group clearly demonstrated that during the human development, the type 1 isoenzyme is not detectable in the fetus, is transiently expressed in newborn skin and scalp, and permanently expressed in skin, especially in sebaceous gland, from the time of puberty. Both isozymes are expressed in the liver, but only after birth. Furthermore, the type 2 isoenzyme is transiently expressed in skin and scalp of newborns and is the predominant 5 $\alpha$ -reductase detectable in fetal genital skin, male accessory sex glands, and in the prostate, including benign prostatic hyperplasia and prostate adenocarcinoma tissues. Expression of type 2 was found in stromal cells of the seminal vesicle as well as in basal epithelial and stromal cells but not in luminal epithelial cells of the normal prostate. It is also of interest to note that its expression was detected in epithelial cells of the epididymis but not in the surrounding stroma. Expression of the type 2 enzyme in these male accessory reproductive tissues is sustained throughout life. These findings are consistent with 5 $\alpha$ -reductase type 1 being responsible for virilization observed during puberty in patients suffering from 5 $\alpha$ -reductase type 2 deficiency, and suggest that the type 2 isozyme may be an initiating factor in development of male pattern baldness, taking into consideration that no qualitative difference in 5 $\alpha$ -reductase type 1 expression was observed between adult balding versus nonbalding scalp (Thigpen *et al.*, 1992b, 1993; Silver *et al.*, 1994a, b).

#### *SRD5A2 polymorphisms and prostate cancer*

The availability of several polymorphisms in both SRD5A1 and SRD5A2 genes provided useful tools to perform genetic association studies for some androgen-sensitive diseases, such as prostate cancer (Jenkins *et al.*, 1991; Labrie *et al.*, 1992; Thigpen *et al.*, 1992b). Prostate cancer has proven to be the most hormone-sensitive cancer to hormonal manipulation. It is not surprising that analyses of genes encoding key proteins involved in androgen biosynthesis and action, led to the observation of a significant association between common genetic variants and a susceptibility to prostate cancer. One of the best candidate genes is the 5 $\alpha$ -reductase type 2 gene (SRD5A2). Indeed, modulation of 5 $\alpha$ -reductase activity may be responsible for some variations in prostate cancer risk among ethnic groups (Ross *et al.*, 1992; Reichardt *et al.*, 1995; Makridakis *et al.*, 1997). Furthermore, one of these variants, A49T has been reported to increase the catalytic activity of this enzyme and is associated with an increased risk of advanced prostate cancer (Makridakis *et al.*, 1999; Jaffe *et al.*, 2000).

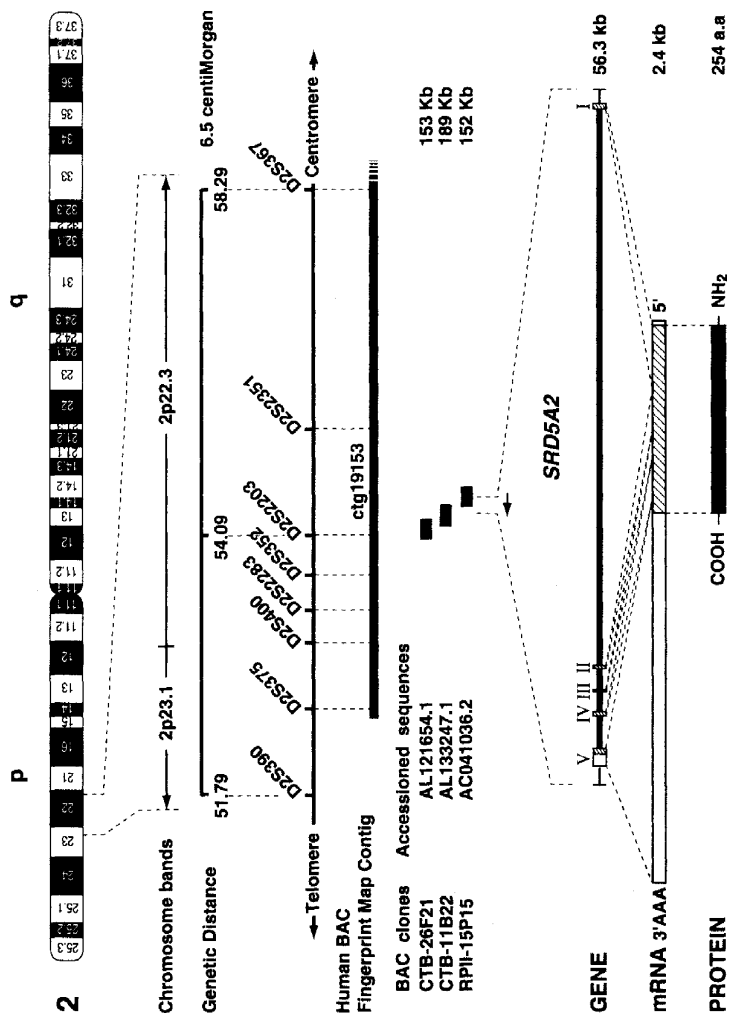
*Male and female isoenzymes of 5 $\alpha$ -reductases*

It has been postulated that the type 1 gene specifies a female isoenzyme, whereas the type 2 gene specifies a male isoenzyme (Mahendroo and Russell, 1999). This hypothesis was suggested taking into account that mutations of the type 1 gene in mice affect reproduction in females by decreasing fecundity and blocking parturition, but has no effect on reproduction in males, whereas mutation of the 5 $\alpha$ -reductase type 2 gene in mice and men prevents proper virilization but does not affect development or reproductive function in females. Indeed, female knockout mice lacking 5 $\alpha$ -reductase type 1 show a parturition defect that is maternal in origin, which is reversed by administration of 5 $\alpha$ -androstan-3 $\alpha$ , 17 $\beta$ -diol (3 $\alpha$ -Adiol) (Mahendroo *et al.*, 1996). Although oogenesis, fertilization, implantation, and placental morphology appear normal in the mutant females, the average litter in homozygous deficient females is 2.7 pups versus 8.0 pups in wild type controls. Fetal loss occurs between gestation days 10.75 and 11.0 concomitantly with a mid-pregnancy surge in placental androgen synthesis and an induction of 5 $\alpha$ -reductase type 1 expression in the decidua of wild type mice. These authors elegantly demonstrated that in the deficient mice, a failure to 5 $\alpha$ -reduce androgens leads to their conversion to estrogens, which in turn causes fetal death in midgestation, thus indicating that the 5 $\alpha$ -reduction of androgens in female animals plays a crucial role in guarding against estrogen toxicity during pregnancy (Mahendroo *et al.*, 1997).

**Clinical features of 5 $\alpha$ -reductase type 2 deficiency**

This rare form of male pseudohermaphroditism was first defined on clinical and genetic grounds by Nowakowski and Lenz in 1961 as *Pseudovaginal perineoscrotal hypospadias* because the infant usually presents a severely hypoplastic penis with the urethra opening on the perineum (Nowakowski and Lenz, 1961). The patients are 46XY males characterized by a female external genitalia at birth and two normally differentiated testes and Wolffian structures that terminate in the vagina. Striking and selective signs of masculinization appear at puberty (Wilson *et al.*, 1993; Forest, 1995; Grumbach and Conte, 1999).

It was later established in 1974 by studies in two families with this syndrome, one in Dallas (Walsh *et al.*, 1974) and the other in the southwestern part of Dominican Republic (Imperato-McGinley *et al.*, 1974), that the disorder is caused by a deficiency in steroid 5 $\alpha$ -reductase. This autosomal recessive disease is now termed steroid 5 $\alpha$ -reductase deficiency. The classic form of this male pseudohermaphroditism in infancy included clitoris-like hypospadiac phallus bound in chordee of variable degree, a bifid scrotum, and a urogenital sinus that opens on the perineum. The normal testes are located in the inguinal canal or the labioscrotal folds. The Wolffian duct structures (i.e., epididymes, vas deferens, seminal vesicles and ejaculatory ducts) are well differentiated and usually terminate in the blind-ending vagina or on the perineum next the urethra if the vaginal pouch is absent. The prostate is hypoplastic. The Müllerian ducts normally regress, a feature critical for the diagnosis (Forest, 1995). Although most patients have been raised as girls, in several families the phallus was so large that affected infants were identified as males with hypospadias and raised as boys and different phenotypes (hypospadias, isolated micropenis) can be seen in the same kindred (Ng *et al.*, 1990; Mendonca *et al.*, 1996; Sinnecker *et al.*, 1996).



**Figure 11.4 Fine chromosomal localization of the SRD5A2 gene.** Location in centimorgan of each marker was determined according to the genetic linkage map of genethon. The human BAC fingerprint map contig was obtained by using the Human Genome Project Working Draft of the Washington University Genome Sequencing Center, St. Louis, MO, USA (<http://genome.ucsc.edu/goldenPath/hgTracks.html>). The clones come from the pieter J. de Jong RPCL-11 libraries (Children's Hospital Oakland—BACPAC Resources Oakland, CA, USA) and Caltech B libraries.



At puberty, plasma T levels increase into adult range, while DHT levels remain disproportionately low but measurable. They exhibit signs of virilization with descent of the testes, muscle mass increase and deepening of the voice, but without gynecomastia. Phallus enlarges to 4–8 cm in length, libido ensues and penile erection may occur (Forest, 1995; Grumbach and Conte, 1999). Furthermore, none of the postpubertal affected males have acne, temporal hair recession and facial and body hair remain often scanty. There is no prostatic tissue. Testis in adult affected males shows Leydig cells hyperplasia and spermatogenesis is incomplete. Whether this abnormality is a direct consequence of the SRD5A2 mutation or the secondary effect of incomplete testicular descent is uncertain (Cai *et al.*, 1994; Johnson *et al.*, 1986). Indeed, semen analyzes of nine patients from the Dominican cohort revealed normal sperm concentration, total count and mobility in one patient with hypospadias and bilaterally descended testes and in a second patient after hypospadias repair (Cai *et al.*, 1994; Katz *et al.*, 1997). Semen volume and viscosity were abnormal in all nine patients. These findings suggest that DHT, in contrast to T, does not play a major role in spermatogenesis.

Females homozygous for deleterious mutations in the SRD5A2 gene are phenotypically normal and undergo normal pubertal maturation except for delayed menarche (Katz *et al.*, 1995; Milewich *et al.*, 1995; Hochberg *et al.*, 1996). However, the importance of 5 $\alpha$ -reductase type 2 expression for the intracrine DHT synthesis in body skin is well illustrated by the observation that these female individuals have an absence of hair on the arms and legs and decreased axillary and pubic hair thus showing the key role of DHT on the growth of body hair (Katz *et al.*, 1995).

The fact that several of these women are the mothers of several children confirms that fertility is normal. It is also of interest to mention that plasma 5 $\alpha$ -dihydroprogesterone levels were normal in two adult women, one a homozygote and the other a compound heterozygote, during the luteal phase, thus suggesting that circulating 5 $\alpha$ -dihydroprogesterone in women is derived principally from the steroid 5 $\alpha$ -reductase type 1 isoenzyme and leaves open the question of whether this steroid plays a physiological role in women (Milewich *et al.*, 1995). As expected heterozygotes for 5 $\alpha$ -reductase deficiency have no clinical manifestation.

### Biological diagnosis

The diagnosis of 5 $\alpha$ -reductase deficiency can be difficult to establish, especially before puberty. The presence of this disorder should be suspected in all prepubertal male pseudohermaphrodites, especially in those with perineoscrotal hypospadias, with or without a blind-ending vagina, in males with hypospadias, in males with microphallus and in males who virilize at puberty without evidence of gynecomastia. Nevertheless, it should be noted that patients suffering from type 3 17P-HSD/KSR deficiency or partial androgen resistance may also virilize at puberty without gynecomastia, but they can be distinguished by DNA analysis or biochemically (Grumbach and Conte, 1999). The biochemical abnormalities characteristic of 5 $\alpha$ -reductase deficiency are clearly described in adults in whom the endocrine characteristics demonstrate the lack of formation of DHT or its metabolites. The diagnosis can be confirmed prepubertally or postpubertally by the detection of an abnormal

plasma T/DHT ratio before or after hCG stimulation. This is easily shown at puberty with a plasma basal T/DHT ratio increased from  $12 \pm 3.1$  (mean  $\pm$  SD) in normal men, to 35 to 84, a diminished urinary ratio of 5 $\alpha$ -reduced to 5 $\beta$ -reduced steroid metabolites, and a decreased urinary androstanediol excretion (Forest, 1995; Grumbach and Conte, 1999). However, it has been reported that this parameter might be normal at birth and become abnormal with age (Odame *et al.*, 1992). At all ages the mutant 5 $\alpha$ -reductase type 2 enzyme causes a decrease in hepatic 5 $\alpha$ -reductase activity leading to low excretion of 5 $\alpha$ -allotetrahydrocortisol (THF) and androsterone allowing biochemical detection of the abnormality (Forest, 1995). The ratio of THF/5 $\alpha$ -THF appears more discriminative in infancy but the latter requires sensitive and specific detection methods (Odame *et al.*, 1992). Alternatively, direct *in vitro* studies demonstrating decreased production of DHT from T in cultured genital skin fibroblasts can also be used to confirm the diagnosis. In fact, DNA analyses of the SRD5A2 gene may be used to establish the molecular diagnostic.

### Molecular diagnosis

SRD5A2 mutations distributed throughout its coding region were identified in individuals suffering from 5 $\alpha$ -reductase type 2 deficiency originating from 76 unrelated families (Table 11.2, Figure 11.5). Eight females (L55Q/L55Q: family 4; G183S/G183S: family 13; R246W/R246W: family 41; R246W/725+1, G $\rightarrow$ T: family 69) have been reported to carry mutations, but they were all asymptomatic. In the first study designed to elucidate the molecular basis of this disorder, it was shown that a complete deletion of the SRD5A2 gene is responsible for this disease in the Sambia tribe of the New Guinea Highlands (Andersson *et al.*, 1991). Thereafter, subsequent studies led to the identification of several single-base mutations including in most of patients missense mutations, whereas splice junction, nonsense and frameshift mutations were less commonly found (Figure 11.5). These mutations were present in approximately 30 different ethnic groups, suggesting a high degree of genetic heterogeneity in this disorder. The presence of a founder effect in geographical isolates of people with a relatively higher coefficient of inbreeding is most likely responsible for the occurrence of this disorder in several populations including for example cohorts originating from Dominican Republic (R246W), Turkey (G196S; 251delA), New Guinea Highlands (complete deletion of the SRD5A2 gene) and Mexico (P212R).

Although 5 $\alpha$ -reductase type 2 deficiency is inherited in an autosomal recessive fashion, in seven well-documented most likely compound heterozygotes, only one mutant allele has been found (Wilson *et al.*, 1993; Boudon *et al.*, 1995; Silver and Russell, 1999; Vilchis *et al.*, 2000), suggesting that the second mutation lies in a region of the gene not yet screened. However, a case (patient 25, Table 11.2) of uniparental disomy has been reported, thus showing an alternate mechanism whereby this disorder can derive from a single parent (Chavez *et al.*, 2000).

Among the 22 missense mutations that have been functionally characterized and enzymatic activity assessed, only 10 mutations were found to retain any residual 5 $\alpha$ -reductase type 2 activity (Wigley *et al.*, 1994). Two of the mutations, G34R and H231R, primarily affected the ability of the enzyme to bind T, whereas the remaining eight mutations,

Table 11.2 Genotype-phenotype relationships of patients with 5 $\alpha$ -reductase type 2 deficiency bearing mutations in the SRD5A2 gene. The ethnic origin, the phenotypic characteristics, reasons for referral and clinical features of patients are indicated as well as a summary of the functional consequence of mutations in the SRD5A2 gene.

Family	Origin	Karyotype	Age at diagnosis	Phenotype or reason for referral	Consanguinity	Case report and mutation report	Mutant alleles	Apparent activity
<i>Homozygotes</i>								
1	Vietnamese	46XY	35 years	Blind-ending vagina, Female phenotype	Yes	(Johnson <i>et al.</i> , 1986)/(Thigpen <i>et al.</i> , 1992b)	G34R	Yes
2	Mexican	46XY	22 years	Perineal hypospadias, Vaginal pouch	No	(Canto <i>et al.</i> , 1997)	G34R	Yes
3	Turkish	46XY	Birth	Clitoral enlargement, Female phenotype	Yes	(Hiort <i>et al.</i> , 1996a)	L55Q	No
4	Lebanese	46XY (8 affected)	10–17 years	Clitoral enlargement, Inguinal testes	Yes	(Hochberg <i>et al.</i> , 1996)	L55Q	No
		46XX	15 years	Spotless skin, No sign of acne or hirsutism	Yes	(Hochberg <i>et al.</i> , 1996)	L55Q	No
5	Algerian	46XY (3 patient)	28 years	Female phenotype	No	(Wigley <i>et al.</i> , 1994)	P59R	No
6	Latvian	46XY	13 years	Clitoromegaly, Female phenotype	Yes	(Jenkins <i>et al.</i> , 1992)/(Thigpen <i>et al.</i> , 1992b)	Y91D	No
7	American French	46XY	20 years	Clitoromegaly, Urogenital sinus with small vagina pouch	No	(Mauvais-Jarvis <i>et al.</i> , 1981)/(Wilson <i>et al.</i> , 1993)	R103X	No predicted activity
8	Mexican-mestizo	46XY	22 years	Perineal hypospadias, vaginal pouch	No	(Vilchis <i>et al.</i> , 2000)	G115D	No
9	Creole-American	46XY	20 years	Clitoromegaly, Blind-ending vagina	Yes	(Goebelsmann <i>et al.</i> , 1975)/(Thigpen <i>et al.</i> , 1992b)	Q126R	No
10	Italian American	46XY	6 years	Perineal hypospadias, Clitoromegaly, Short vaginal pouch	No	(Saenger <i>et al.</i> , 1978)/(Wilson <i>et al.</i> , 1993)	Q126R	No
11	Portuguese	46XY	30 years	Perineal hypospadias, vaginal pouch communicated with the urethra	No	(Deslypere <i>et al.</i> , 1985)/(Wilson <i>et al.</i> , 1993)	Q126R	No

Family	Origin	Karyotype	Age at diagnosis	Phenotype or reason for referral	Consanguinity	Case report and mutation report	Mutant alleles	Apparent activity
12	French	46XY	3 months	Female phenotype	No	(Boudon <i>et al.</i> , 1995a)	Q126R	No
13	Brazilian (Black)	46XY	31 years	Perineal hypospadias, Vaginal pouch	Yes	(Thigpen <i>et al.</i> , 1992b)	G183S	Yes
14	Greek	46XX	41 years	Normal female	Yes	(Milewich <i>et al.</i> , 1995)	G196S	Yes
15	American Turkish	46XY	9 months	Perineal hypospadias	No	(Carpenter <i>et al.</i> , 1990)/ (Thigpen <i>et al.</i> , 1992b)	G196S	Yes
18	Turkish	46XY	Birth	Perineal hypospadias, Micropenis of 1 cm	Yes	(Hiort <i>et al.</i> , 1996a; Hiort <i>et al.</i> , 1996b)	G196S	Yes
19	Mexican-mestizo	46XY	Birth	Perineal hypospadias, Micropenis of 1 cm	Yes	(Sinnecker <i>et al.</i> , 1996)	G196S	Yes
20	Pakistani	46XY	13 years	Clitoromegaly	No	(Chavez <i>et al.</i> , 2000)	E197D	No
			26 years	Ambiguous genitalia with palpable labial testes	Yes	(Anwar <i>et al.</i> , 1997)	E200L	?
21	Mexican-mestizo	46XY (2 affected)	17 years	Perineal hypospadias	No	(Mendez <i>et al.</i> , 1995)/ (Canto <i>et al.</i> , 1997)	A207D	No
22	Mexican-mestizo	46XY	11 years 14 years	Perineal hypospadias	No	(Mendez <i>et al.</i> , 1995)/ (Canto <i>et al.</i> , 1997)	P212R	No
23	Mexican-mestizo	46XY (2 affected)	36 years 32 years	Perineal hypospadias	No	(Mendez <i>et al.</i> , 1995)/ (Canto <i>et al.</i> , 1997)	P212R	No
24	Mexican	46XY	Not available	Female phenotype	No	(Wigley <i>et al.</i> , 1994)	P212R	No
25	American-mestizo	46XY	6 months	Female phenotype	No	(Vilchis <i>et al.</i> , 2000)	P212R	No
26	American Indian	46XY	16 years	Clitoromegaly, Blind-ending vaginal pouch	No	(Johnson <i>et al.</i> , 1986)/ (Thigpen <i>et al.</i> , 1992b)	L224P	No
27	Brazilian (Creole)	46XY	6 years	Perineal hypospadias, Female phenotype	Yes	(Thigpen <i>et al.</i> , 1992b)	R227X	No predicted activity

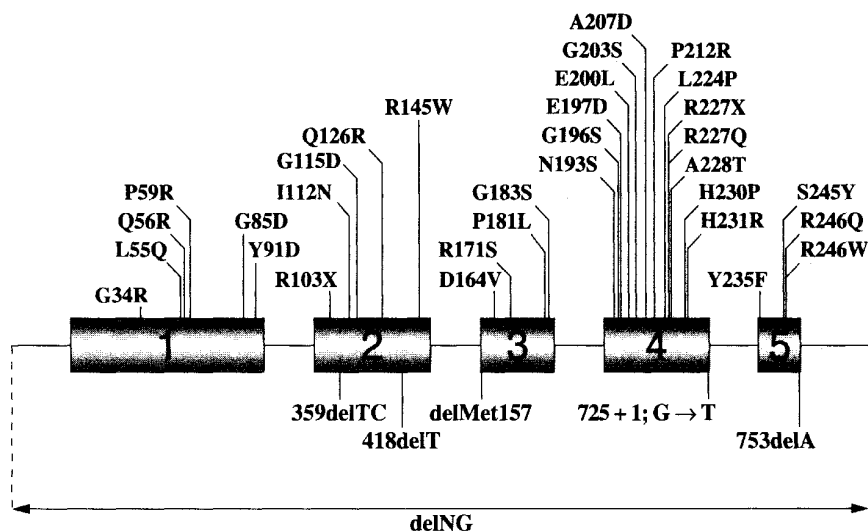
28	Mexican American	46XY	16 years	Clitoromegaly, Female phenotype	No	(Johnson <i>et al.</i> , 1986)/ (Thigpen <i>et al.</i> , 1992b)	R227X	No predicted activity
30	Vietnamese	46XY	5 years	Small penis 2.5 cm	No	(Sinnecker <i>et al.</i> , 1996)	R227Q	?
32	Eritrean	46XY	8 years	Perineal hypospadias	No	(Sinnecker <i>et al.</i> , 1996)	R227Q	?
		46XY	4 years	Female phenotype	Yes	(Sinnecker <i>et al.</i> , 1996)	A228T	?
33	Polish	46XY	14 years	Blind vaginal pouch	No	(Boudon <i>et al.</i> , 1995b)	H231R	Yes
34	German	46XY	22 years	Clitoromegaly	No	(Hiort <i>et al.</i> , 1996a)	H231R	Yes
35	German	46XY	12 years	Clitoromegaly	No	(Sinnecker <i>et al.</i> , 1996)	H231R	Yes
36	Italian	46XY	4 months	Clitoromegaly, Female phenotype	No	(Forti <i>et al.</i> , 1996)	H231R	Yes
37	Caucasian	46XY	31 years	Female phenotype	?	(Wigley <i>et al.</i> , 1994)	Y235F	?
38	Pakistani	46XY	Birth	Perineal hypospadias	Yes	(Jenkins <i>et al.</i> , 1992)/ (Thigpen <i>et al.</i> , 1992b)	R246Q	Yes
39	Pakistani	46XY	9.5 years	Perineal hypospadias, Microphallus	No	(Thigpen <i>et al.</i> , 1992b)	R246Q	Yes
40	Mexican-mestizo	46XY	32 years	Perineal hypospadias	No	(Vilchis <i>et al.</i> , 2000)	R246Q	Yes
41	Dominican Republic kindred (29 families)	46XY (47 affected)	Not available	44 affected male with clitoris-like phallus, bifid scrotum, urogenital sinus	Yes	(Imperato-McCinley <i>et al.</i> , 1974; Imperato-McCinley <i>et al.</i> , 1979; Jenkins <i>et al.</i> , 1992; Peterson <i>et al.</i> , 1977)/ (Thigpen <i>et al.</i> , 1992a)	R246W	Yes
42	Brazilian (White)	46XX (3 affected)	33, 35, 36 years	Normal female	Yes	(Katz <i>et al.</i> , 1995)/ (Thigpen <i>et al.</i> , 1992b)	R246W	Yes
43	Papua New Guinea (Simbari)	46XY	17 years	Microphallus, Bilateral cryptorchidism	Yes	(Thigpen <i>et al.</i> , 1992b)		
	Anga linguistic group of the Eastern Highlands)		Not available	Perineal hypospadias, Clitoromegaly	Yes	(Imperato-McCinley <i>et al.</i> , 1991; Jenkins <i>et al.</i> , 1992)/ (Andersson <i>et al.</i> , 1991)	delNG complete deletion of SRD5A2	No

Family	Origin	Karyotype	Age at diagnosis	Phenotype or reason for referral	Consanguinity	Case report and Mutation report	Mutant alleles	Apparent activity
44	Pakistani	46XY	5 years	Perineal hypospadias, Stretched penis of 4 cm	Yes	(Price <i>et al.</i> , 1984)/ (Thigpen <i>et al.</i> , 1992b)	725 + 1, G → T	?
45	Pakistani	46XY	6 years	Perineal hypospadias	Yes	(Odame <i>et al.</i> , 1992)/ (Wilson <i>et al.</i> , 1993)	725 + 1, G → T	?
46	Turkish	46XY	2 years	Clitoromegaly, Female phenotype	Yes	(Boudon <i>et al.</i> , 1995a)	delMet157	No predicted activity
47	Turkish	46XY	Birth	Clitoromegaly, Marked labial fusion	Yes	(Hiort <i>et al.</i> , 1996a)	delMet157	No predicted activity
48	Turkish	46XY	14 years	Perineal hypospadias, Clitoromegaly	Yes	(Sinnecker <i>et al.</i> , 1996)	delMet157	No predicted activity
49	Turkish	46XY	14.5 years	Clitoromegaly, Marked labial fusion	Yes	(Sinnecker <i>et al.</i> , 1996)	delMet157	No predicted activity
50	Maltese	46XY	Not available	Not available	No	(Thigpen <i>et al.</i> , 1992b)	359delTC	No predicted activity
51	Maltese	46XY	Not available	Not available	No	(Wilson <i>et al.</i> , 1993)	359delTC	No predicted activity
52	Turkish kindred (4 distinct families)	46XY (8 affected)	6 months to adult	Perineal hypospadias, Bifid scrotum	Yes	(Akgun <i>et al.</i> , 1986; Can <i>et al.</i> , 1998)/ (Can <i>et al.</i> , 1998)	753delA	No
<i>Compound heterozygotes</i>								
53	Mexican-American	46XY	17 years	Bilateral testes and epididymis	No	(Thigpen <i>et al.</i> , 1992b)	G34R/G115D	Yes/No

54	Silician	46XY	16 years	No blind vaginal pouch, Presence of testis	No	(Imperato-McGinley <i>et al.</i> , 1980; Jenkins <i>et al.</i> , 1992)/ (Thigpen <i>et al.</i> , 1992b)	G34R/R171S	Yes/Yes
55	Jordanian	46XY	28 years	Perineal hypospadias, Palpable testes	Unknown	(Thigpen <i>et al.</i> , 1992b)	L55Q/Q56R	No/No
56	Mexican-mestizo	46XY	8 years	Perineal hypospadias	No	(Vilchis <i>et al.</i> , 2000)	G85D/G115D	?/No
57	Russian	46XY	Birth	Female phenotype	No	(Thigpen <i>et al.</i> , 1992b)/ (Wilson <i>et al.</i> , 1993)	Y91D/E197D	No/No
58	German	46XY	Birth	Female phenotype	Unknown	(Hiort <i>et al.</i> , 1996a)	I112N/Q126R	?/No
59	German	46XY	4 months	Female phenotype	No	(Sinnecker <i>et al.</i> , 1996)	I112N/Q126R	?/No
60	Mexican	46XY	17 years	Perineal hypospadias	No	(Canto <i>et al.</i> , 1997)	G115D/G203S	No/?
61	Brazilian (Creole)	46XY	29 years	Perineal hypospadias, Microphallus, Testes in inguinal canal	No	(Thigpen <i>et al.</i> , 1992b)	Q126R/D164V	No/No
62	Brazilian	46XY	14 years	Perineal hypospadias, blind-ending vaginal pouch, testes in bifid scrotum	No	(Mendonca <i>et al.</i> , 1987)/ (Wilson <i>et al.</i> , 1993)	Q126R/N193S	No/Yes
63	Brazilian (caucasian)	46XY	14 years	Palpable gonads, Vaginal introitus	No	(Ferraz <i>et al.</i> , 1999)	Q126R/418delT	No/No predicted activity
64	Maltese	46XY	10 years	Perineal hypospadias	No	(Jenkins <i>et al.</i> , 1992; Price <i>et al.</i> , 1984)/ (Thigpen <i>et al.</i> , 1992b)	R171S/359delTC	Yes/No predicted activity
65	Italian American	46XY	7 years	Blind ending vaginal pouch	No	(Saenger <i>et al.</i> , 1978)/ (Wilson <i>et al.</i> , 1993)	P181L/H230P	Yes/No
66	Swedish	46XY (3 affected)	16, 14, 10 years	Clitoromegaly, Short vaginal pouch	No	(Nordenskjöld and Ivarsson, 1998)	G196S/H231R	Yes/Yes
				Perineal hypospadias, Microphallus	No			

Family	Origin	Karyotype	Age at diagnosis	Phenotype or reason for referral	Consequence	Case report and mutation report	Mutant alleles	Apparent activity
67	Mexican-mestizo	46XY	17 years	Perineal hypospadias, Gonads in scrotum, Gynecomastia	No	(Chavez <i>et al.</i> , 2000)	E197D/P212R	No/No
68	Austrian	46XY	16 years	Perineal hypospadias, Clitoromegaly	No	(Bartsch <i>et al.</i> , 1987; Johnson <i>et al.</i> , 1986)/ (Thigpen <i>et al.</i> , 1992b)	A207D/R246Q	No/Yes
69	Pakistani	46XX	Not available	Normal female	Yes	(Milewich <i>et al.</i> , 1995)	R246W/725 + 1, G → T	No/?
<i>Heterozygotes/?</i>								
70	New Guinean	46XY	Not available	Data not available	Unknown	(Wigley <i>et al.</i> , 1994; Wilson <i>et al.</i> , 1993)	R145W/?	Yes
71	Polish	46XY	16 years	Clitoromegaly, Strong masculine body proportions	No	(Boudon <i>et al.</i> , 1995a)	N198S/?	Yes
72	African American	46XY	13 years	Blind-ending vagina, bilateral inguinal masses	No	(Jenkins <i>et al.</i> , 1992; Walsh <i>et al.</i> , 1974)/ (Thigpen <i>et al.</i> , 1992b)	H231R/?	Yes
73	American (White)	46XY	Birth	Microphallus with perineal urethra, Testes palpable in labia	No	(Thigpen <i>et al.</i> , 1992b)	H231R/?	Yes
74	American	46XY	Not available	Perineal hypospadias	No	(Silver and Russell, 1999)	H231R/?	Yes
75	Italian	46XY	57 years	Normal fertile male	No	(Vilchus <i>et al.</i> , 2000)	S245Y/?	?
76	African American	46XY (2 affected)	6 years and 10 years	Clitoromegaly, Blind vaginal pouch, Gonads palpable in inguinal canals	No	(Fisher <i>et al.</i> , 1978; Jenkins <i>et al.</i> , 1992; Leshin <i>et al.</i> , 1978)/ (Thigpen <i>et al.</i> , 1992b)	R246Q/?	Yes





**Figure 11.5** Representation of all the mutations identified to date in the SRD5A2 gene in individuals suffering from 5 $\alpha$ -reductase type 2 deficiency. All the missense mutations are shown in the upper panel, while the frameshift and splice-junction mutations are shown below.

R145W, R171S, P181L, G183S, N193S, G196S, R246W, and R246Q, decreased the affinity of the enzyme for NADPH.

The absence of detectable activity in transfected cells expressing mutant cDNAs could be due to a rapid turnover of the mutant protein or to inactivity of the enzyme. The mutant enzymes could be divided into three classes, including those with: (1) very short half-lives ranging from 1 to 5 hours (L55Q, Y91D, Q126R, P181L, G196S, E197D, A207D, P212R, L224P, H230P, R246Q, R246W); (2) intermediate half-lives ranging from 5 to 15 hours (P59R, R145W, N193S); and (3) essentially normal half-lives ranging from 15 to 30 hours (G34R, Q56R, G115D, D164 V, R171S, G183S, H231R)(Wigley *et al.*, 1994). There was no apparent correlation between protein half-life and enzyme activity showing the complexity of the genotype-phenotype relationships.

Mutations that affect T binding map to the two ends of the type 2 isoenzyme suggesting that the substrate binding domain is composed of non-linear determinants, whereas those that affect NADPH binding map throughout the conserved carboxy-terminal half of the protein (Figure 11.5). The mechanism by which the G34R mutation disrupts substrate binding was not determined but, it seems likely that the effect is caused by the substitution of the bulky and charged Arg for the smaller Gly residue (Wigley *et al.*, 1994). Furthermore, it is of interest to note that amino acids involved in finasteride binding have been mapped in the type 1 isoenzyme to residues 26–29 (Thigpen *et al.*, 1992a). The comparable amino acids in the type 2 isoenzyme are located between residues 21 and 24 and are thus close to the G34R mutation. It remains to be determined how and if the amino-terminal segment interacts with His 231 to form the substrate binding domain. Both rat and human type 1 and type 2 isoenzymes have two or three histidines at this location and mutations in two of these

residues, 230 and 231, inactivate or impair, respectively, the type 2 isoenzyme (Wigley *et al.*, 1994).

Seven distinct amino acids that contribute to NADPH cofactor binding are located in the carboxyl-terminal half of the type 2 isoenzyme. The molecular mechanism by which mutation of these residues alters the affinity for the cofactor is not yet elucidated, while these mutant enzymes have a near normal affinity for T. Despite this dependence on NADPH, the amino acid sequences of the rat and human isoenzyme do not contain consensus adenine dinucleotide-binding sequences or NADPH selective residues identified in other reductase enzymes (Perham *et al.*, 1991). This absence suggests that the cofactor-binding domain of the 5 $\alpha$ -reductase may represent a novel structure, a hypothesis in keeping with the diversity and location of mutations that affect NADPH binding in the type 2 isoenzyme. These eight mutant proteins have also a shorter half-life in both fibroblasts and transfected cells (Russell and Wilson, 1994). In agreement with these results, numerous studies indicate that NADPH stabilizes enzyme activity in some tissue extracts. Taken together, these results strengthen the conclusion that mutations affecting NADPH cofactor binding directly disrupt this domain of the enzyme and that variations in the intracellular levels of cofactor could regulate turnover of 5 $\alpha$ -reductase.

A systematic screening of penile skin specimens obtained at surgery during hypospadias repair showed that 7 out of 81 (8.6%) carried a mutation in at least one SRD5A2 allele (A49T, L113V, H231R), while two patients were homozygotes for the A49T mutations (Silver and Russell, 1999). Neither A49T nor the L113V mutation has been reported in association with 5 $\alpha$ -reductase type 2 deficiency. The mutation A49T was found in five of these patients and it was generally present in less severe forms of hypospadias. In view of this finding it has been suggested that a partial deficiency of 5 $\alpha$ -reductase activity and inadequate levels of DHT in fetal urethra may be sufficient to cause the phenotype of hypospadias without other clinical features of 5 $\alpha$ -reductase deficiency. On the other hand, A49T has been reported to increase the catalytic activity of this enzyme and is associated with an increased risk of advanced prostate cancer (Makridakis *et al.*, 1999; Jaffe *et al.*, 2000). Further studies will be needed to better understand the role, if any, of A49T in the etiology of hypospadias.

## Management

Early diagnosis of 5 $\alpha$ -reductase deficiency is important for the assignment of sex in the affected infant. As discussed in previous sections, although the majority of missense mutations in SDR5A2 gene almost completely abolish the enzyme activity, some mutant proteins showing a low but sufficient residual activity, have been detected in 46XY individuals with sufficient masculinization of the external genitalia at birth to be assigned a male gender (Wilson *et al.*, 1993; Grumbach and Conte, 1999). If diagnosed early, infants can now be treated specifically with DHT with a good response (Carpenter *et al.*, 1990; Odame *et al.*, 1992). For example, in a nine-month-old affected infant who had been assigned a male gender at birth, a four-month treatment with DHT, 25mg/day, caused an increase of stretched phallus length from 1.8 to 3.8cm. Hypospadias repair was done and a second course of DHT was administered without consequence (Carpenter *et al.*, 1990). Based on

their experience with males with microphallus, Grumbach and Conte suggested that it would be prudent to maintain the phallic length at or above the 50% range for age by using additional short courses of DHT until the onset of puberty and spontaneous phallus development (Grumbach and Conte, 1999). Thus, if the diagnosis is made during the neonatal period, a male sex of rearing is recommended and androgen therapy is used to enhance penile growth and facilitate surgical repair of the genitalia (Forest, 1995). However, it should be noted that the safety of long term use of androgen therapy remains to be established. In such patients, further virilization can be expected at puberty and fertility is theoretically possible. Indeed, paternity by intrauterine insemination with sperm from a man with 5 $\alpha$ -reductase-2 deficiency demonstrates that spermatogenesis can occur in this disorder (Katz *et al.*, 1997).

Those diagnosed later in life and who have an unambiguous female gender identity, should undergo gonadectomy before puberty to preclude or stop the partial virilization, receive estrogen replacement therapy at the time of puberty to promote feminization, and, when appropriate, vaginoplasty should be done either by surgical or medical means (Griffin and Wilson, 1992). Thus, in those individuals who elect to lead life as women, the management is similar to that in women with testicular feminization and allied syndromes, but it should be undertaken only after careful psychiatric and psychological evaluation (Griffin and Wilson, 1992; Wilson *et al.*, 1993).

Although the assignment of the sex of rearing was in the past based in large part on the degree of anatomical and functional impairment, this decision in 5 $\alpha$ -reductase deficiency is complicated by the fact that reversal of gender behavior is common in those raised as women (Griffin and Wilson, 1992; Wilson *et al.*, 1993). Discovery of this phenomenon has been served to reinvigorate the argument as to relative roles of biological determinants and psychological factors in the development of gender identity (Wilson *et al.*, 1993). It is of interest to note that a similar phenomenon has also been reported in patients suffering from 17 $\beta$ -HSD/KSR type 3 deficiency, 45X/46XY gonadal dysgenesis, and in other male pseudohermaphrodites in which the diagnosis is not clear, whereas such a behavioral change is not a feature of subjects bearing mutations in the androgen receptor in which gender behavior usually conforms to the predominant anatomical development and hence to gender assignment (Wilson *et al.*, 1993). The reports of gender reversal in 5 $\alpha$ -reductase deficiency are striking taking into consideration that the phenomenon occurs in different ethnic groups and in different social settings. It has been suggested that androgen action in the brain (T or DHT) *in utero*, during the neonatal period, and/or at puberty has an impact on the determination of male gender identity in this disorder that is so pervasive that it can override female sex assignment and female rearing (Imperato-McGinley *et al.*, 1979; Wilson *et al.*, 1993). Whether these individuals undergo a true change of gender identity, as contrasted to a change in gender role behavior has been previously discussed in details (Wilson *et al.*, 1993). In view of these observations, male assignment of neonatally diagnosed patients appears appealing, especially in affected subjects with ambiguous or hypoplastic male genitalia (Ng *et al.*, 1990; Mendonca *et al.*, 1996; Sinnecker *et al.*, 1996; Ferraz *et al.*, 1999). Nevertheless, the mechanism by which androgens, including DHT, influence sexual behavior is perhaps the most challenging of all unresolved problems of 5 $\alpha$ -reductase deficiency (Wilson *et al.*, 1993).

## REFERENCES

- Adamski, J., Husen, B., Marks, F. and Jungblut, P.W. (1992) Purification and properties of oestradiol 17 $\beta$ -dehydrogenase extracted from cytoplasmic vesicles of porcine endometrial cells. *Biochem. J.* **288**, 375–381.
- Ademola-Akese, F., Meyer, W.J. and Migeon, C.J. (1977) Male pseudohermaphroditism with gynaecomastia due to testicular 17-ketosteroid reductase deficiency. *Clin. Endocrinol. (Oxf.)* **7**, 443–452.
- Akgun, S., Ertel, N.H., Imperato-McGinley, J., Sayli, B.S. and Shackleton, C. (1986) Familial male pseudohermaphroditism due to 5 $\alpha$ -reductase deficiency in a Turkish village. *Am. J. Med.* **81**, 267–274.
- Andersson, S., Berman, D.M., Jenkins, E.P. and Russell, D.W. (1991) Deletion of steroid 5 $\alpha$ -reductase 2 gene in male pseudohermaphroditism. *Nature* **354**, 159–161.
- Andersson, S., Geissler, W.M., Wu, L., Davis, D.L., *et al.* (1996) Molecular genetics and pathophysiology of 17 $\beta$ -hydroxy steroid dehydrogenase 3 deficiency. *J. Clin. Endocrinol. Metab.* **81**, 130–136.
- Andersson, S. and Russell, D.W. (1990) Structural and biochemical properties of cloned and expressed human and rat steroid 5 $\alpha$ -reductases. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3640–3644.
- Anwar, R., Gilbey, S.G., New, J.P. and Markham, A.F. (1997) Male pseudohermaphroditism resulting from a novel mutation in the human steroid 5 $\alpha$ -reductase type 2 gene (SRD5A2). *Mol. Pathol.* **50**, 51–52.
- Arnholt, I.J., Mendonca, B.B., Diaz, J.A., Nogueira, C., *et al.* (1988) Prepubertal male pseudohermaphroditism due to 17-ketosteroid reductase deficiency: diagnostic value of a hCG test and lack of HLA association. *J. Endocrinol. Invest.* **11**, 319–322.
- Azzi, Z., Rehse, P., Zhu, D.-W., Campbell, R.L., Labrie, F. and Lin, S.-X. (1996) Crystal structure of human estrogenic 17 $\beta$ -hydroxysteroid dehydrogenase complexed with 17 $\beta$ -estradiol at 2.3 Å resolution. *Nature Struct. Biol.* **3**, 665–668.
- Azziz, N., Maxwell, M.M., St Jacques, B. and Brenner, B.M. (1993) Downregulation of Ke6 a novel gene encoded within the major histocompatibility complex, in murine polycystic kidney disease. *Mol. Cell. Biol.* **13**, 1847–1853.
- Bartsch, G., Decristoforo, A. and Schweikert, U. (1987) Pseudovaginal perineoscrotal hypospadias. Clinical, endocrinological and biochemical characterization of a patient. *Eur. Urol.* **13**, 386–389.
- Bilbao, J.R., Loridan, L., Audi, L., Gonzalo, E. and Castano, L. (1998) A novel missense (R80W) mutation in 17 $\beta$ -hydroxysteroid dehydrogenase type 3 gene associated with male pseudohermaphroditism. *Eur. J. Endocrinol.* **139**, 330–333.
- Biswas, M.G. and Russell, D.W. (1997) Expression cloning and characterization of oxidative 17 $\beta$ - and 3 $\alpha$ -hydroxysteroid dehydrogenase from rat and human prostate. *J. Biol. Chem.*, 15959–15966.
- Boehmer, A.L., Brinkmann, A.O., Sandkuijl, L.A., Halley, D.J., *et al.* (1999) 17 $\beta$ -hydroxysteroid dehydrogenase-3 deficiency: diagnosis, phenotypic variability, population genetics, and worldwide distribution of ancient and *de novo* mutations. *J. Clin. Endocrinol. Metab.* **84**, 4713–4721.
- Boudon, C., Lobaccaro, J.M., Lumbroso, S., Ogur, G., Ocal, G., Belon, C. and Sultan, C. (1995a) A new deletion of the 5 $\alpha$ -reductase type 2 gene in a Turkish family with 5 $\alpha$ -reductase deficiency. *Clin. Endocrinol. (Oxf.)* **43**, 183–188.
- Boudon, C., Lumbroso, S., Lobaccaro, J.M., Szarras-Czapnik, M., Romer, T.E., Garandeau, P., Montoya, P. and Sultan, C. (1995b) Molecular study of the 5 $\alpha$ -reductase type 2 gene in three European families with 5 $\alpha$ -reductase deficiency. *J. Clin. Endocrinol. Metab.* **80**, 2149–2153.

- Cai, L.Q., Fratianni, C.M., Gautier, T. and Imperato-McGinley, J. (1994) Dihydrotestosterone regulation of semen in male pseudohermaphrodites with 5 $\alpha$ -reductase-2 deficiency. *J. Clin. Endocrinol. Metab.* **79**, 409–414.
- Calemard-Michel, L., Bertrand, A., Forest, M., Chatelain, P., Rappaport, R., Tourniaire, J., Nivelon, J., David, M. and Morel, Y. (1996a) Le deficit en 17-ceto-reductase est bien du a des mutations du gene 17 $\beta$ -hydroxysteroid deshydrogenase type 3: Etude de 8 familles. *Ann. Endocrinol.* **57**, 357.
- Calemard-Michel, L., Jaubert, F., Lortat-Jacob, S., Meduri, G., Morel, Y. and Rappaport, R. (1996b) Pitfalls in early diagnosis of 17 $\beta$ -hydroxy steroid dehydrogenase deficiency: clinical, histological and molecular studies. *Horm. Res.* **46** (Suppl. 2), 93.
- Calemard-Michel, L., Mebarki, F., Bertrand, A., Forest, M., Chatelain, P. and Morel, Y. (1996c) Homozygous 702insAA and R80Q mutations of the 17 $\beta$ -hydroxysteroid dehydrogenase type 3 gene in two families with 17 $\beta$ -HSD deficiency. In *10th International Congress of Endocrinology*, Vol. 1, San Francisco.
- Can, S., Zhu, Y.S., Cai, L.Q., Ling, Q., Katz, M.D., Akgun, S., Shackleton, C.H. and ImperatoMcGinley, J. (1998) The identification of 5 $\alpha$ -reductase-2 and 17 $\beta$ -hydroxysteroid dehydrogenase-3 gene defects in male pseudohermaphrodites from a Turkish kindred. *J. Clin. Endocrinol. Metab.* **83**, 560–569.
- Canto, P., Vilchis, F., Chavez, B., Mutchinick, O., Imperato-McGinley, J., Perez-Palacios, G., UlloaAguirre, A. and Mendez, J.P. (1997) Mutations of the 5 $\alpha$ -reductase type 2 gene in eight Mexican patients from six different pedigrees with 5 $\alpha$ -reductase-2 deficiency. *Clin. Endocrinol. (Oxf.)* **46**, 155–160.
- Carpenter, T.O., Imperato-McGinley, J., Boulware, S.D., Weiss, R.M., Shackleton, C., Griffin, J.E. and Wilson, J.D. (1990) Variable expression of 5 $\alpha$ -reductase deficiency: presentation with male phenotype in a child of Greek origin. *J. Clin. Endocrinol. Metab.* **71**, 318–322.
- Casey, M.L., MacDonald, P.C. and Andersson, S. (1994) 17 $\beta$ -Hydroxy steroid dehydrogenase type 2: Chromosomal assignment and progestin regulation of gene expression in human endometrium. *J. Clin. Invest.* **94**, 2135–2141.
- Chavez, B., Valdez, E. and Vilchis, F. (2000) Uniparental disomy in steroid 5 $\alpha$ -reductase 2 deficiency. *J. Clin. Endocrinol. Metab.* **85**, 3147–3150.
- David, M., Nicod, P., de Peretti, E., Forest, M. and Jeune, M. (1975) Pseudo-hermaphrodisme masculin par deficit testiculaire en 17-ceto-reductase. In *Société de Pédiatrie du Sud de la France*.
- David, M., Calemard-Michel, L., Morel, Y. and Forest, M. (1997) Difficulties in the diagnosis of 17 $\beta$ -hydroxysteroid dehydrogenase deficiency. Mimiking the androgen insensitivity syndrome (AIS) in early infancy. In *European Society for Pediatric Endocrinology*, Vol. 48 (Suppl. 12),  $\beta$ . 111, Stockholm, Sweden.
- Deslypere, J.P., Coucke, W., Robbe, N. and Vermeulen, A. (1985) 5 $\alpha$ -reductase deficiency: an infrequent cause of male pseudohermaphroditism. *Acta Clin. Belg.* **40**, 240–246.
- Deyashiki, Y., Ohshima, K., Nakanishi, M., Sato, K., Matsuura, K. and Hara, A. (1995) Molecular cloning and characterization of mouse estradiol 17 $\beta$ -dehydrogenase (A-specific), a member of the aldo-ketoreductase family. *J. Biol. Chem.* **270**, 10461–10467.
- Dufort, I., Rheault, P., Huang, X.F., Soucy, P. and Luu-The, V. (1999) Characteristics of a highly labile human type 5 17 $\beta$ -hydroxysteroid dehydrogenase. *Endocrinology* **140**, 568–574.
- Durocher, F., Morissette, J., Labrie, Y., Labrie, F. and Simard, J. (1995) Mapping of the HSD17B2 gene encoding type II 17 $\beta$ -hydroxysteroid dehydrogenase close to D16S422 on chromosome 16q24.1-q24.2. *Genomics* **25**, 724–726.
- El-Alfy, M., Luu-The, V., Huang, X.F., Berger, L., Labrie, F. and Pelletier, G. (1999) Localization of type 5 17 $\beta$ -hydroxy steroid dehydrogenase, 3 $\beta$ -hydroxysteroid dehydrogenase, and androgen

- receptor in the human prostate by *in situ* hybridization and immunocytochemistry. *Endocrinology* **140**, 1481–1491.
- Ferraz, L.F., Mathias Baptista, M.T., Maciel-Guerra, A.T., Junior, G.G. and Hackel, C. (1999) New frameshift mutation in the 5 $\alpha$ -reductase type 2 gene in a Brazilian patient with 5 $\alpha$ -reductase deficiency. *Am. J. Med. Genet.* **87**, 221–225.
- Fisher, L.K., Kogut, M.D., Moore, R.J., Goebelsmann, U., Weitzman, J.J., Isaacs, H., Jr., Griffin, J.E. and Wilson, J.D. (1978) Clinical, endocrinological, and enzymatic characterization of two patients with 5 $\alpha$ -reductase deficiency: evidence that a single enzyme is responsible for the 5 $\alpha$ -reduction of cortisol and testosterone. *J. Clin. Endocrinol. Metab.* **47**, 653–664.
- Fomitcheva, J., Baker, M.E., Anderson, E., Lee, G.Y. and Aziz, N. (1998) Characterization of Ke 6, a new 17 $\beta$ -hydroxysteroid dehydrogenase, and its expression in gonadal tissues. *J. Biol. Chem.* **273**, 22664–22671.
- Forest, M., Bertrand, A., Sainmont, C., Betuel, H. and Morel, Y. (1989) Familial 17-ketosteroid reductase deficiency (17KSR-D): two affected members in a kindred of six. In *Proceedings of the 71st Annual Meeting of the Endocrine Society*, Seattle.
- Forest, M.G. (1995) Diagnosis and Treatment of Disorders of Sexual Development. In *Endocrinology* (Edited by Degroot, L.J.), Vol. 2, pp. 1901–1937. W.B. Saunders Company, Montreal.
- Forest, M.G., de Peretti, E. and Campo-Paysaa, A. (1979) Familial case of male pseudohermaphroditism due to 17-ketoreductase defect: late diagnosis in the aunt of a patient with the same defect (author's transl). *Ann. Endocrinol.* **40**, 545–546.
- Forest, M.G., Mebatki, F., David, A., Bureau, L. and Morel, Y. (1995) Diagnosis of partial 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) questioned: lessons from long-term study and molecular biology. *Horm. Res.* **44**, 55.
- Forti, G., Falchetti, A., Santoro, S., Davis, D.L., Wilson, J.D. and Russell, D.W. (1996) Steroid 5 $\alpha$ -reductase 2 deficiency: virilization in early infancy may be due to partial function of mutant enzyme. *Clin. Endocrinol. (Oxf.)* **44**, 477–482.
- Geissler, W.M., Davis, D.L., Wu, L., Bradshaw, K.D., Patel, S., Mendonca, B.B., Elliston, K.O., Wilson, J.D., Russell, D.W. and Andersson, S. (1994) Male pseudohermaphroditism caused by mutations of testicular 17 $\beta$ -hydroxysteroid dehydrogenase 3. *Nat. Genet.* **7**, 34–39.
- Ghosh, D., Pletnev, V.Z., Zhu, D.W., Wawrzak, Z., Duax, W.L., Pangborn, W., Labrie, F. and Lin, S.X. (1995) Structure of human estrogenic 17 $\beta$ -hydroxysteroid dehydrogenase at 2.20 Å resolution. *Structure* **3**, 503–513.
- Givens, J.R., Wiser, W.L., Summitt, R.L., Kerber, I.J., Andersen, R.N., Pittaway, D.E. and Fish, S.A. (1974) Familial male pseudohermaphroditism without gynecomastia due to deficient testicular 17-ketosteroid reductase activity. *N. Engl. J. Med.* **291**, 938–944.
- Goebelsmann, U., Hall, T.D., Paul, W.L. and Stanczyk, F.Z. (1975) *In vitro* steroid metabolic studies in testicular 17 $\beta$ -reduction deficiency. *J. Clin. Endocrinol. Metab.* **41**, 1136–1143.
- Griffin, J.E. and Wilson, J.D. (1992) Disorders of sexual differentiation (Edited by Walsh, P.C., Retik, A.B., Stamey, T.A. and Vaughan, J.), WB Saunders, Philadelphia, pp. 1509–1542.
- Grumbach, M. and Conte, F. (1999) Disorders of sex differentiation. In *Williams Textbook of Endocrinology* (Edited by Wilson, J.D. and Foster, D.W.), Saunders, pp. 1303–1425.
- Hiort, O., Sinnecker, G.H., Willenbring, H., Lehnert, A., Zollner, A. and Struve, D. (1996a) Non-isotopic single strand conformation analysis of the 5 $\alpha$ -reductase type 2 gene for the diagnosis of 5 $\alpha$ -reductase deficiency. *J. Clin. Endocrinol. Metab.* **81**, 3415–3418.
- Hiort, O., Willenbring, H., Albers, N., Hecker, W., Engert, J., Dibbelt, L. and Sinnecker, G.H. (1996b) Molecular genetic analysis and human chorionic gonadotropin stimulation tests in the diagnosis of prepubertal patients with partial 5 $\alpha$ -reductase deficiency. *Eur. J. Pediatr.* **155**, 445–451.

- Hochberg, Z., Chayen, R., Reiss, N., Falik, Z., Makler, A., Munichor, M., Farkas, A., Goldfarb, H., Ohana, N. and Hiort, O. (1996) Clinical, biochemical, and genetic findings in a large pedigree of male and female patients with 5 $\alpha$ -reductase 2 deficiency. *J. Clin. Endocrinol. Metab.* **81**, 2821–2827.
- Imperato-McGinley, J., Guerrero, L., Gautier, T. and Peterson, R.E. (1974) Steroid 5 $\alpha$ -reductase deficiency in man: an inherited form of male pseudohermaphroditism. *Science* **186**, 1213–1215.
- Imperato-McGinley, J., Peterson, R.E., Gautier, T. and Sturla, E. (1979) Androgens and the evolution of male-gender identity among male pseudohermaphrodites with 5 $\alpha$ -reductase deficiency. *N. Engl. J. Med.* **300**, 1233–1237.
- Imperato-McGinley, J., Peterson, R.E., Leshin, M., Griffin, J.E., Cooper, G., Draghi, S., Berenyi, M. and Wilson, J.D. (1980) Steroid 5 $\alpha$ -reductase deficiency in a 65-year-old male pseudohermaphrodite: the natural history, ultrastructure of the testes, and evidence for inherited enzyme heterogeneity. *J. Clin. Endocrinol. Metab.* **50**, 15–22.
- Imperato-McGinley, J., Akgun, S., Ertel, N.H., Sayli, B. and Shackleton, C. (1987) The coexistence of male pseudohermaphrodites with 17-ketosteroid reductase deficiency and 5 $\alpha$ -reductase deficiency within a Turkish kindred. *Clin. Endocrinol. (Oxf.)* **27**, 135–143.
- Imperato-McGinley, J., Miller, M., Wilson, J.D., Peterson, R.E., Shackleton, C. and Gajdusek, D.C. (1991) A cluster of male pseudohermaphrodites with 5 $\alpha$ -reductase deficiency in Papua New Guinea. *Clin. Endocrinol. (Oxf.)* **34**, 293–298.
- Jaffe, J.M., Malkowicz, S.B., Walker, A.H., MacBride, S., Peschel, R., Tomaszewski, J., Van Arsdalen, K., Wein, A.J. and Rebbeck, T.R. (2000) Association of SRD5A2 genotype and pathological characteristics of prostate tumors. *Cancer Res.* **60**, 1626–1630.
- Jenkins, E.P., Andersson, S., Imperato-McGinley, J., Wilson, J.D. and Russell, D.W. (1992) Genetic and pharmacological evidence for more than one human steroid 5 $\alpha$ -reductase. *J. Clin. Invest.* **89**, 293–300.
- Jenkins, E.P., Hsieh, C.L., Milatovich, A., Normington, K., Berman, D.M., Francke, U. and Russell, D.W. (1991) Characterization and chromosomal mapping of a human steroid 5 $\alpha$ -reductase gene and pseudogene and mapping of the mouse homologue. *Genomics* **11**, 1102–1112.
- Johnson, L., George, F.W., Neaves, W.B., Rosenthal, I.M., Christensen, R.A., et al. (1986) Characterization of the testicular abnormality in 5 $\alpha$ -reductase deficiency. *J. Clin. Endocrinol. Metab.* **63**, 1091–1099.
- Katz, M.D., Cai, L.Q., Zhu, Y.S., Herrera, C., DeFillo-Ricart, M., Shackleton, C.H. and Imperato-McGinley, J. (1995) The biochemical and phenotypic characterization of females homozygous for 5 $\alpha$ -reductase-2 deficiency. *J. Clin. Endocrinol. Metab.* **80**, 3160–3167.
- Katz, M.D., Kligman, L., Cai, L.Q., Zhu, Y.S., Fratianni, C.M., Zervoudakis, L., Rosenwaks, Z. and Imperato-McGinley, J. (1997) Paternity by intrauterine insemination with sperm from a man with 5 $\alpha$ -reductase-2 deficiency. *N. Engl. J. Med.* **336**, 994–997.
- Khanna, M., Qin, K.N., Wang, R.W. and Cheng, K.C. (1995) Substrate specificity, gene structure, and tissue-specific distribution of multiple human 3 $\alpha$ -hydroxysteroid dehydrogenases. *J. Biol. Chem.* **270**, 20162–20168.
- Kikuti, Y.Y., Tamiya, G., Ando, A., Chen, L., Kmra, M., Ferreira, E., Tsuji, K., Trowdale, J. and Inoko, H. (1997) Physical mapping 220kb centromeric of the human MHC and DNA sequence analysis of the 43kb segment including the RING-1, HKE6, and HKE4 genes. *Genomics* **42**, 422–435.
- Knorr, D., Bidlingmaier, F. and Engelhardt, D. (1973) Reifenstein's syndrome, a 17 $\beta$ -hydroxysteroidoxydoreductase deficiency? *Acta Endocrinol.* **173** (Suppl.), 37.

- Kohn, G., Lasch, E.E., El-Shawwa, R., Elrayyes, E., Litvin, Y. and Rosler, A. (1985) Male pseudohermaphroditism due to 17 $\beta$ -hydroxysteroid dehydrogenase deficiency (17 $\beta$ -HSD) in a large Arab kinship. Studies on the natural history of the defect. *J. Ped. Endocrinol.* **1**, 29–37.
- Krazeisen, A., Breitling, R., Imai, K., Fritz, S., Moller, G. and Adamski, J. (1999) Determination of cDNA, gene structure and chromosomal localization of the novel human 17 $\beta$ -hydroxysteroid dehydrogenase type 7. *FEBS Lett.* **460**, 373–379.
- Labrie, F., Belanger, A., Luu-The, V., *et al.* (1999) Role of DHEA transformation into androgens and estrogens in peripheral intracrine tissues. In *DHEA a comprehensive review* (Edited by Thijssen, J. and Nieuwenhuys, H.), Parthenon Publishing, New York, pp. 69–103.
- Labrie, F., Luu-The, V., Lin, S.X., Simard, J., Labrie, C., El-Alfy, M., Pelletier, G. and Belanger, A. (2000) Intracrinology: role of the family of 17 $\beta$ -hydroxysteroid dehydrogenases in human physiology and disease. *J. Mol. Endocrinol.* **25**, 1–16.
- Labrie, F., Sugimoto, Y., Luu-The, V., Simard, J., Lachance, Y., Bachvarov, D., Leblanc, G., Durocher, F. and Paquet, N. (1992) Structure of human type II 5 $\alpha$ -reductase gene. *Endocrinology* **131**, 1571–1573.
- Labrie, Y., Durocher, F., Lachance, Y., Turgeon, C., Simard, J., Labrie, C. and Labrie, F. (1995) The human type II 17 $\beta$ -hydroxysteroid dehydrogenase gene encodes two alternatively spliced mRNA species. *DNA Cell. Biol.* **14**, 849–861.
- Leenders, F., Prescher, G., Dolez, V., Begue, A., de Launoit, Y. and Adamski, J. (1996) Assignment of human 17 $\beta$ -hydroxysteroid dehydrogenase IV to chromosome 5q2 by fluorescence *in situ* hybridization. *Genomics* **37**, 403–404.
- Leshin, M., Griffin, J.E. and Wilson, J.D. (1978) Hereditary male pseudohermaphroditism associated with an unstable form of 5 $\alpha$ -reductase. *J. Clin. Invest.* **62**, 685–691.
- Li, J., Biswas, M.G., Chao, A., Russell, D.W. and Chory, J. (1997) Conservation of function between mammalian and plant steroid 5 $\alpha$ -reductases. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3554–3559.
- Lin, H.K., Jez, J.M., Schlegel, B.P., Peehl, D.M., Pachter, J.A. and Penning, T.M. (1997) Expression and characterization of recombinant type 2 3 $\alpha$ -hydroxysteroid dehydrogenase (HSD) from human prostate: demonstration of bifunctional 3 $\alpha$ /17 $\beta$ -HSD activity and cellular distribution [published erratum appears in *Mol. Endocrinol.* (1999) **12**, 1763]. *Mol. Endocrinol.* **11**, 1971–1984.
- Luu-The, V., Dufort, L., Pelletier, G. and Labrie, F. (2001) Type 5 17 $\beta$ -hydroxysteroid dehydrogenase: its role in the formation of androgens in women. *Mol. Cell. Endocrinol.* **171**, 77–82.
- Luu-The, V., Labrie, C., Simard, J., Lachance, Y., Zhao, H.F., Couet, J., Leblanc, G. and Labrie, F. (1990) Structure of two in tandem human 17 $\beta$ -hydroxysteroid dehydrogenase genes. *Mol. Endocrinol.* **4**, 268–275.
- Luu-The, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, J., Strickler, R. and Labrie, F. (1989) Full length cDNA structure and deduced amino acid sequence of human 3 $\beta$ -hydroxy-5-ene steroid dehydrogenase. *Mol. Endocrinol.* **3**, 1310–1312.
- Mahendroo, M.S., Cala, K.M., Landrum, D.P. and Russell, D.W. (1997) Fetal death in mice lacking 5 $\alpha$ -reductase type 1 caused by estrogen excess. *Mol. Endocrinol.* **11**, 917–927.
- Mahendroo, M.S., Cala, K.M. and Russell, D.W. (1996) 5 $\alpha$ -reduced androgens play a key role in murine parturition. *Mol. Endocrinol.* **10**, 380–392.
- Mahendroo, M.S. and Russell, D.W. (1999) Male and female isoenzymes of steroid 5 $\alpha$ -reductase. *Rev. Reprod.* **4**, 179–183.
- Makridakis, N., Ross, R.K., Pike, M.C., Chang, L., *et al.* (1997) A prevalent missense substitution that modulates activity of prostatic steroid 5 $\alpha$ -reductase. *Cancer Res.* **57**, 1020–1022.



- Makridakis, N.M., Ross, R.K., Pike, M.C., Crocitto, L.E., *et al.* (1999) Association of mis-sense substitution in SRD5A2 gene with prostate cancer in African-American and Hispanic men in Los Angeles, USA. *Lancet* **354**, 975–978.
- Martel, C., Rhéaume, E., Takahashi, M., Trudel, C., Couet, J., Luu-The, V., Simard, J. and Labrie, F. (1992) Distribution of 17 $\beta$ -hydroxysteroid dehydrogenase gene expression and activity in rat and human tissues. *J. Steroid. Biochem. Mol. Biol.* **41**, 597–603.
- Mauvais-Jarvis, P., Kuttann, F., Mowszowicz, I. and Wright, F. (1981) Different aspects of 5 $\alpha$ -reductase deficiency in male pseudohermaphroditism and hypothyroidism. *Clin. Endocrinol. (Oxf.)* **14**, 459–469.
- Mendez, J.P., Ulloa-Aguirre, A., Imperato-McGinley, J., Bruggmann, A., *et al.* (1995) Male pseudohermaphroditism due to primary 5 $\alpha$ -reductase deficiency: variation in gender identity reversal in seven Mexican patients from five different pedigrees. *J. Endocrinol. Invest.* **18**, 205–213.
- Mendonca, B.B., Batista, M.C., Arnhold, I.J., Nicolau, W., *et al.* (1987) Male pseudohermaphroditism due to 5 $\alpha$ -reductase deficiency associated with gynecomastia. *Rev. Hosp. Clin. Fac. Med. Sao Paulo* **42**, 66–68.
- Mendonca, B.B., Arnhold, I.J., Bloise, W., Andersson, S., Russell, D.W. and Wilson, J.D. (1999) 17 $\beta$ -hydroxysteroid dehydrogenase 3 deficiency in women. *J. Clin. Endocrinol. Metab.* **84**, 802–804.
- Mendonca, B.B., Inacio, M., Costa, E.M., Arnhold, I.J., Silva, F.A., Nicolau, W., Bloise, W., Russell, D.W. and Wilson J.D. (1996) Male pseudohermaphroditism due to steroid 5 $\alpha$ -reductase-2 deficiency. Diagnosis, psychological evaluation, and management. *Medicine (Baltimore)* **75**, 64–76.
- Miettinen, M.M., Mustonen, M.V., Poutanen, M.H., Isomaa, V.V. and Vihko, R.K. (1996) Human 17 $\beta$ -hydroxysteroid dehydrogenase type 1 and type 2 isoenzymes have opposite activities in cultured cells and characteristic cell- and tissue-specific expression. *Biochem. J.* **314**, 839–845.
- Milewich, L., Mendonca, B.B., Arnhold, I., Wallace, A.M., Donaldson, M.D., Wilson, J.D. and Russell, D.W. (1995) Women with steroid 5 $\alpha$ -reductase-2 deficiency have normal concentrations of plasma 5 $\alpha$ -dihydroprogesterone during the luteal phase. *J. Clin. Endocrinol. Metab.* **80**, 3136–3139.
- Moghrabi, N., Head, J.R. and Andersson, S. (1997) Cell type-specific expression of 17 $\beta$ -hydroxysteroid dehydrogenase type 2 in human placenta and fetal liver. *J. Clin. Endocrinol. Metab.* **82**, 3872–3878.
- Moghrabi, N., Hughes, I.A., Dunaif, A. and Andersson, S. (1998) Deleterious missense mutations and silent polymorphism in the human 17 $\beta$ -hydroxy steroid dehydrogenase 3 gene (HSD17B3). *J. Clin. Endocrinol. Metab.* **83**, 2855–2860.
- Moore, R.J., Griffin, J.E. and Wilson, J.D. (1975) Diminished 5 $\alpha$ -reductase activity in extracts of fibroblasts cultured from patients with familial incomplete male pseudohermaphroditism, type 2. *J. Biol. Chem.* **250**, 7168–7172.
- Moore, R.J. and Wilson, J.D. (1976) Steroid 5 $\alpha$ -reductase in cultured human fibroblasts. Biochemical and genetic evidence for two distinct enzyme activities. *J. Biol. Chem.* **251**, 5895–5900.
- Morisette, J., Durocher, F., Leblanc, J.F., Normand, T., Labrie, F. and Simard, J. (1996) Genetic linkage mapping of the human steroid 5 $\alpha$ -reductase type 2 gene (SRD5A2) close to D2S352 on chromosome region 2p23→p22. *Cytogenet. Cell Genet.* **73**, 304–307.
- Mustonen, M., Poutanen, M., Chotteau-Lelievre, A., de Launoit, Y., Isomaa, V., Vainio, S., Vihko, R. and Vihko, P. (1997) Ontogeny of 17 $\beta$ -hydroxy steroid dehydrogenase type 2 mRNA expression in the developing mouse placenta and fetus. *Mol. Cell. Endocrinol.* **134**, 33–40.

- Mustonen, M.V., Poutanen, M.H., Kellokumpu, S., de Launoit, Y., Isomaa, V.V., Vihko, R.K. and Vihko, P.T. (1998) Mouse 17 $\beta$ -hydroxy steroid dehydrogenase type 2 mRNA is predominantly expressed in hepatocytes and in surface epithelial cells of the gastrointestinal and urinary tracts. *J. Mol. Endocrinol.* **20**, 67–74.
- Neher, R. and Kahnt, F. (1965) Gonadal steroid biosynthesis *in vitro* in four cases of testicular feminization. In *Androgens in normal and pathological conditions* (Edited by Exley, V.), Vol. Excerpta Medica International Congress Series no. 101, Amsterdam, pp. 130–136.
- Ng, W.K., Taylor, N.F., Hughes, I.A., Taylor, J., Ransley, P.G. and Grant, D.B. (1990) 5 $\alpha$ -reductase deficiency without hypospadias. *Arch. Dis. Child.* **65**, 1166–1167.
- Nokelainen, P., Peltoketo, H., Vihko, R. and Vihko, P. (1998) Expression cloning of a novel estrogenic mouse 17 $\beta$ -hydroxysteroid dehydrogenase/17-ketosteroid reductase (m17HSD7), previously described as a prolactin receptor-associated protein (PRAP) in rat. *Mol. Endocrinol.* **12**, 1048–1059.
- Nordenskjold, A. and Ivarsson, S.A. (1998) Molecular characterization of 5 $\alpha$ -reductase type 2 deficiency and fertility in a Swedish family. *J. Clin. Endocrinol. Metab.* **83**, 3236–3238.
- Novikov, D., Dieuaide-Noubhani, M., Vermeesch, J.R., Fournier, B., Mannaerts, G.P. and Van Veldhoven, P.P. (1997) The human peroxisomal multifunctional protein involved in bile acid synthesis: Activity measurement, deficiency in Zellweger syndrome and chromosome mapping. *Biochim. Biophys. Acta* **1360**, 229–240.
- Nowakowski, H. and Lenz, W. (1961) Genetic aspects in male hypogonadism. *Recent. Prog. Horm. Res.* **17**, 53–95.
- Odame, I., Donaldson, M.D., Wallace, A.M., Cochran, W. and Smith, P.J. (1992) Early diagnosis and management of 5 $\alpha$ -reductase deficiency. *Arch. Dis. Child.* **67**, 720–723.
- Opitz, J.M., Simpson, J.L., Sarto, G.E., Summitt, R.L., New, M. and German, J. (1972) Pseudovaginal perineoscrotal hypospadias. *Clin. Genet.* **3**, 1–26.
- Pang, S.Y., Softness, B., Sweeney, W.J. and New, M.I. (1987) Hirsutism, polycystic ovarian disease, and ovarian 17-ketosteroid reductase deficiency. *N. Engl. J. Med.* **316**, 1295–1301.
- Pelletier, G., Luu-The, V., Tetu, B. and Labrie, F. (1999) Immunocytochemical localization of type 5 17 $\beta$ -hydroxy steroid dehydrogenase in human reproductive tissues. *J. Histochem. Cytochem.* **47**, 731–738.
- Peltoketo, H., Isomaa, V., Maentausta, O. and Vihko, R. (1988) Complete amino acid sequence of human placental 17 $\beta$ -hydroxy steroid dehydrogenase deduced from cDNA. *FEBS Lett.* **239**, 73–77.
- Peltoketo, H., Isomaa, V. and Vihko, R. (1992) Genomic organization and DNA sequences of human 17 $\beta$ -hydroxysteroid dehydrogenase genes and flanking regions. Localization of multiple Alu sequences and putative cis-acting elements. *Eur. J. Biochem.* **209**, 459–466.
- Peltoketo, H., Luu-The, V., Simard, J. and Adamski, J. (1999) 17 $\beta$ -hydroxysteroid dehydrogenase (HSD)/17-ketosteroid reductase (KSR) family; nomenclature and main characteristics of the 17HSD/KSR enzymes. *J. Mol. Endocrinol.* **23**, 1–11.
- Peretti, D., Saez, J. and Bertrand, J. (1970) Familial male pseudohermaphroditism (MPH) due to 17-ketosteroid reductase defect: *in vitro* study and testicular incubation. In *Excerpta Medica International Congress Series* no. 210, Amsterdam, p. 205.
- Perham, R.N., Scrutton, N.S. and Berry, A. (1991) New enzymes for old: redesigning the coenzyme and substrate specificities of glutathione reductase. *Bioessays* **13**, 515–525.
- Peterson, R.E., Imperato-McGinley, J., Gautier, T. and Sturla, E. (1977) Male pseudohermaphroditism due to steroid 5 $\alpha$ -reductase deficiency. *Am. J. Med.* **62**, 170–191.

- Price P., Wass J.A., Griffin J.E., Leshin M., *et al.* (1984) High dose androgen therapy in male pseudohermaphroditism due to 5 $\alpha$ -reductase deficiency and disorders of the androgen receptor. *J. Clin. Invest.* **74**, 1496–1508.
- Qin, Y.M., Poutanen, M.H., Helander, H.M., Kvist, A.P., *et al.* (1997) Peroxisomal multifunctional enzyme of  $\beta$ -oxidation metabolizing D-3-hydroxyacyl-CoA esters in rat liver: molecular cloning, expression and characterization. *Biochem. J.* **321**, 21–28.
- Quigley, C. (1998) Disorders of sex determination and differentiation. In *Principles of molecular medicine* (Edited by Jameson J.), Totowa, NJ, pp. 527–559.
- Reichardt, J.K., Makridakis, N., Henderson, B.E., Yu, M.C., Pike, M.C. and Ross, R.K. (1995) Genetic variability of the human SRD5A2 gene: implications for prostate cancer risk. *Cancer Res.* **55**, 3973–3975.
- Rheume, E., Sanchez, R., Mebarki, F., Gagnon, E., *et al.* (1995) Identification and characterization of the G15D mutation found in a male patient with 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) deficiency: Alteration of the putative NAD-binding domain of type II 3 $\beta$ -HSD. *Biochemistry* **34**, 2893–2900.
- Rommens, J.M., Durocher, F., McArthur, J., Tonin, P., *et al.* (1995) Generation of a transcription map at the HSD17B locus centromeric to BRCA1 at 17q21. *Genomics* **28**, 530–542.
- Rosler, A., Belanger, A. and Labrie, F. (1992) Mechanisms of androgen production in male pseudohermaphroditism due to 17 $\beta$ -hydroxysteroid dehydrogenase deficiency. *J. Clin. Endocrinol. Metab.* **75**, 773–778.
- Rosler, A. and Kohn, G. (1983) Male pseudohermaphroditism due to 17 $\beta$ -hydroxy steroid dehydrogenase deficiency: studies on the natural history of the defect and effect of androgens on gender role. *J. Steroid. Biochem.* **19**, 663–674.
- Rosler, A., Silverstein, S. and Abeliovich, D. (1996) A (R80Q) mutation in 17 $\beta$ -hydroxysteroid dehydrogenase type 3 gene among Arabs of Israel is associated with pseudohermaphroditism in males and normal asymptomatic females. *J. Clin. Endocrinol. Metab.* **81**, 1827–1831.
- Ross, R.K., Bernstein, L., Lobo, R.A., Shimizu, H., Stanczyk, F.Z., Pike, M.C. and Henderson, B.E. (1992) 5 $\alpha$ -reductase activity and risk of prostate cancer among Japanese and US white and black males. *Lancet* **339**, 887–889.
- Russell, D.W. and Wilson, J.D. (1994) Steroid 5 $\alpha$ -reductase: two genes/two enzymes. *Annu. Rev. Biochem.* **63**, 25–61.
- Saenger, P., Goldman, A.S., Levine, L.S., Korth-Schutz, S., Muecke, E.G., Katsumata, M., Doberne, Y. and New, M.I. (1978) Prepubertal diagnosis of steroid 5 $\alpha$ -reductase deficiency. *J. Clin. Endocrinol. Metab.* **46**, 627–634.
- Saez, J.M., de Peretti, E., Morera, A.M., David, M. and Bertrand, J. (1971) Familial male pseudohermaphroditism with gynecomastia due to a testicular 17-ketosteroid reductase defect. I. Studies *in vivo*. *J. Clin. Endocrinol. Metab.* **32**, 604–610.
- Saez, J.M., Morera, A.M., de Peretti, E. and Bertrand, J. (1972) Further *in vivo* studies in male pseudohermaphroditism with gynecomastia due to a testicular 17-ketosteroid reductase defect (compared to a case of testicular feminization). *J. Clin. Endocrinol. Metab.* **34**, 598–600.
- Sasano, H., Frost, A.R., Saitoh, R., Harada, N., Poutanen, M., Vihko, R., Bulun, S.E., Silverberg, S.G. and Nagura, H. (1996) Aromatase and 17 $\beta$ -hydroxysteroid dehydrogenase type 1 in human breast carcinoma. *J. Clin. Endocrinol. Metab.* **81**, 4042–4046.
- Scrutton, N.S., Berry, A. and Perham, R.N. (1990) Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature* **343**, 38–43.
- Siiteri, P.K. and Wilson, J.D. (1974) Testosterone formation and metabolism during male sexual differentiation in the human embryo. *J. Clin. Endocrinol. Metab.* **38**, 113–125.

- Silver, R.I. and Russell, D.W. (1999) 5 $\alpha$ -reductase type 2 mutations are present in some boys with isolated hypospadias. *J. Urol.* **162**, 1142–1145.
- Silver, R.I., Wiley, E.L., Davis, D.L., Thigpen, A.E., Russell, D.W. and McConnell, J.D. (1994a) Expression and regulation of steroid 5 $\alpha$ -reductase 2 in prostate disease. *J. Urol.* **152**, 433–437.
- Silver, R.I., Wiley, E.L., Thigpen, A.E., Guileyardo, J.M., McConnell, J.D. and Russell, D.W. (1994b) Cell type specific expression of steroid 5 $\alpha$ -reductase 2. *J. Urol.* **152**, 438–442.
- Simard, J., Rheaume, E., Sanchez, R., Laflamme, N., *et al.* (1993) Molecular basis of congenital adrenal hyperplasia due to 3 $\beta$ -hydroxysteroid dehydrogenase deficiency. *Mol. Endocrinol.* **7**, 716–728.
- Sinnecker, G.H., Hiort, O., Dibbelt, L., Albers, N., *et al.* (1996) Phenotypic classification of male pseudohermaphroditism due to steroid 5 $\alpha$ -reductase-2 deficiency. *Am. J. Med. Genet.* **63**, 223–230.
- The, V.L., Labrie, C., Zhao, H.F., Couet, J., *et al.* (1989) Characterization of cDNAs for human estradiol 17 beta-dehydrogenase and assignment of the gene to chromosome 17: evidence of two mRNA species with distinct 5'-termini in human placenta. *Mol. Endocrinol.* **3**, 1301–1309.
- Thigpen, A.E., Davis, D.L., Gautier, T., Imperato-McGinley, J. and Russell, D.W. (1992a) Brief report: the molecular basis of steroid 5 $\alpha$ -reductase deficiency in a large Dominican kindred. *N. Engl. J. Med.* **327**, 1216–1219.
- Thigpen, A.E., Davis, D.L., Milatovich, A., Mendonca, B.B., Imperato-McGinley, J., Griffin, J.E., Francke, U., Wilson, J.D. and Russell, D.W. (1992b) Molecular genetics of steroid 5 $\alpha$ -reductase 2 deficiency. *J. Clin. Invest.* **90**, 799–809.
- Thigpen, A.E., Silver, R.I., Guileyardo, J.H., Casey, M.L., McConnell, J.D. and Russell, D.W. (1993) Tissue distribution and ontogeny of steroid 5 $\alpha$ -reductase isozyme expression. *J. Clin. Invest.* **92**, 903–910.
- Toscano, V., Balducci, R., Bianchi, P., Mangiantini, A. and Sciarra, F. (1990) Ovarian 17-ketosteroid reductase deficiency as a possible cause of polycystic ovarian disease. *J. Clin. Endocrinol. Metab.* **71**, 288–292.
- Tourniaire, J., Laubie, B., Saez, J.M., Leung, T.K., Perrin, J., Dutrieux, N. and Guinet, P. (1973) Pseudohermaphroditisme male familial par deficit testiculaire en 17-cetosteroide-reductase. In *Annual Endocrine*, Vol. 34, Paris, pp. 461–465.
- van Grunsven, E.G., van Berkel, E., Ijlst, L., Vreken, P., *et al.* (1998) Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2128–2133.
- Vilchis, F., Mendez, J.P., Canto, P., Lieberman, E. and Chavez, B. (2000) Identification of missense mutations in the SRD5A2 gene from patients with steroid 5 $\alpha$ -reductase 2 deficiency. *Clin. Endocrinol. (Oxf.)* **52**, 383–387.
- Virdis, R., Saenger, P., Senior, B. and New, M.I. (1978) Endocrine studies in a pubertal male pseudohermaphrodite with 17-ketosteroid reductase deficiency. *Acta Endocrinol. (Copenh.)* **87**, 212–224.
- Walsh, P.C., Madden, J.D., Harrod, M.J., Goldstein, J.L., MacDonald, P.C. and Wilson, J.D. (1974) Familial incomplete male pseudohermaphroditism, type 2. Decreased dihydrotestosterone formation in pseudovaginal perineoscrotal hypospadias. *N. Engl. J. Med.* **291**, 944–949.
- Wigley, W.C., Prihoda, J.S., Mowszowicz, I., Mendonca, B.B., New, M.I., Wilson, J.D. and Russell, D.W. (1994) Natural mutagenesis study of the human steroid 5 $\alpha$ -reductase 2 isozyme. *Biochemistry* **33**, 1265–1270.
- Wilson, J. (1982) Gonadal hormone and sexual behavior. In *Clinical Neuroendocrinology* (Edited by Besser, G.M. and L.M.), Vol. 2, Academic Press, New York, pp. 1–29.

- Wilson, J.D. (1972) Recent studies on the mechanism of action of testosterone. *N. Engl. J. Med.* **287**, 1284–1291.
- Wilson, J.D. (1975) Dihydrotestosterone formation in cultured human fibroblasts. Comparison of cells from normal subjects and patients with familial incomplete male pseudohermaphroditism, Type 2. *J. Biol. Chem.* **250**, 3498–3504.
- Wilson, J.D., Griffin, J.E. and Russell, D.W. (1993) Steroid 5 $\alpha$ -reductase-2 deficiency. *Endocr. Rev.* **14**, 577–593.
- Wilson, J.D. and Lasnitzki, I. (1971) Dihydrotestosterone formation in fetal tissues of the rabbit and rat. *Endocrinology* **89**, 659–668.
- Wilson, S.C., Hodgins, M.B. and Scott, J.S. (1987) Incomplete masculinization due to a deficiency of 17 $\beta$ -hydroxysteroid dehydrogenase: comparison of prepubertal and peripubertal siblings. *Clin. Endocrinol. (Oxf.)* **26**, 459–469.
- Wit, J.M., van Hooff, C.O., Thijssen, J.H. and Van den Brande, J.L. (1988) *In vivo* and *in vitro* studies in a 46XY phenotypically female infant with 17-ketosteroid reductase deficiency. *Horm. Metab. Res.* **20**, 367–374.
- Zhang, Y., Word, R.A., Fesmire, S., Carr, B.R. and Rainey, W.E. (1996) Human ovarian expression of 17 $\beta$ -hydroxy steroid dehydrogenase types 1, 2 and 3. *J. Clin. Endocrinol. Metab.* **81**, 3594–3598.
- Zurbrugg, R.P. (1974) Inborn errors in testosterone biosynthesis with special reference to 17-oxosteroid reductase deficiency (1–6). *Helv. Paediatr. Acta* **34** (Suppl.), 63–77.

## 12.

# STEROID METABOLISM IN PERIPHERAL TISSUES

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The tissue-specific expression of enzymes involved in peripheral steroid hormone metabolism has been shown to be a crucial aspect of hormone action. For corticosteroid hormone action, two isozymes of 11 $\beta$ -hydroxysteroid dehydrogenase (types 1 and 2) interconvert hormonally active cortisol and inactive cortisone. For sex steroid hormone action, a related family of short-chain alcohol dehydrogenases (17 $\beta$ -HSD) interconvert active and inactive estrogens and androgens. The remit of this chapter is to discuss these enzymes in terms of their function and the consequence of genetic defects within the genes which encode them.

KEY WORDS: 11 $\beta$ -hydroxysteroid dehydrogenase, glucocorticoid, mineralocorticoid, cortisol, AME, 17 $\beta$ -hydroxysteroid dehydrogenase.

### INTRODUCTION

When considering hormone action in its broadest terms, each member of the thyroid hormone and steroid hormone receptor superfamily has a “pre-receptor” signaling pathway in place in the form of an enzyme responsible for activation or inactivation of the hormone in question. Thus 5'-deiodinase, vitamin D 1 $\alpha$ -hydroxylase, retinoic acid isomerase, in turn regulate the access of the “active” ligands, tri-iodothyronine, 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub>, and 9-*cis* retinoic acid to the thyroid hormone, vitamin D and retinoid receptors respectively. For sex steroid hormone action this role is served by a series of enzymes, 5 $\alpha$ -reductase, aromatase and the 17 $\beta$ -hydroxysteroid dehydrogenases (17 $\beta$ -HSD). Genetic defects in 5 $\alpha$ -reductase and aromatase result in striking phenotypes which are discussed elsewhere (see Chapters 10 and 11). With the exception of the type 3 isozyme of 17 $\beta$ -HSD, to date, there are no clinical phenotypes associated with deficiencies in the other 17 $\beta$ -HSD isozymes, but these fascinating enzymes will be briefly discussed in this chapter. However, the principal remit of this chapter will be to address a related hydroxysteroid dehydrogenase, 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) which plays a crucial role in determining both glucocorticoid and mineralocorticoid hormone action. The biological activity of any glucocorticoid in part relates to the presence of a hydroxyl group at position C-11 of the steroid structure. Cortisol (compound F), and the principal glucocorticoid in rodents,

corticosterone (compound B), are active steroids whereas cortisone (compound E) and 11-dehydrocorticosterone (compound A), possessing a C-11 keto group, are inactive. Thus any tissue expressing 11 $\beta$ -HSD can regulate the exposure of "active" glucocorticoid to that tissue. Early studies, evaluating the interconversion of F to E demonstrated significant amounts of 11 $\beta$ -HSD activity in human placenta (Osinski, 1960), kidney (Jenkins, 1966) and liver (Bush, 1969), though the "set-point" of the enzyme varied, with oxidative activity (F to E) predominating in the placenta and kidney and reductive (E to F) in the liver. As will be discussed this is explained by the activity of two distinct isozymes of 11 $\beta$ -HSD, a predominantly reductive type 1 enzyme and an oxidative type 2 enzyme.

### 11 $\beta$ -HSD ENZYMOLOGY

The 11 $\beta$ -HSD enzymes belong to the short chain alcohol dehydrogenase superfamily (SCAD). Over 120 members have been documented in the protein databases based on consensus sequences. An N-terminal hydrophobic region of up to 100 residues often precedes the cofactor binding domain (Krozowski, 1992) and this region is thought to play a role in anchoring the protein in the endoplasmic reticulum. SCAD members share a common protein folding arrangement of  $\alpha$ -helices and  $\beta$ -strands ( $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$ ) $\times$ 2 to form a Rossmann fold for cofactor binding. The Gly-X-X-X-Gly-X-Gly motif is highly conserved in the cofactor binding domain and mutagenesis studies with a variety of NAD(P)H binding enzymes show that nucleotide specificity is determined by surrounding residues. The active site of these enzymes contain invariant Tyr and Lys residues, although adjacent Ser are also highly conserved. An analysis of active site motifs in SCAD family members showed that 48% contain the YZX(ST)K motif, 30% the Y(ST)X(ST)K and 14% Y (ST)XZK, where X is any residue and Z denotes residues other than Ser or Thr. Coincidentally, the active site contained in type 1 11 $\beta$ -HSD (YSASK) is the most common active site motif, occurring 9% of the time.

### 11 $\beta$ -hydroxysteroid dehydrogenase type 1

In the laboratories of Monder and White, an 11 $\beta$ -hydroxysteroid dehydrogenase was purified from rat liver, an antiserum raised against the protein and used to clone a rat cDNA (Lakshmi and Monder, 1988; Monder and Lakshmi, 1989, 1990; Agarwal *et al.*, 1989). This enzyme is microsomal (Ozols, 1995), NADP-dependent and behaves as a dehydrogenase in a cell free system. Subsequently this enzyme was named type 1 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSDI). Homogeneous enzyme gave rectilinear Eadie plots and Km constants of 1.83 $\pm$ 0.06 $\mu$ M for corticosterone and 17.3 $\pm$ 2.24 $\mu$ M for cortisol. Subsequently, 11 $\beta$ -HSDI cDNA's have been published for the human (Tannin *et al.*, 1991), mouse (Rajan *et al.*, 1995) squirrel monkey (Moore *et al.*, 1993) and sheep (Yang *et al.*, 1992).

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The value of the  $K_m$  determined for  $11\beta$ -HSD1 dehydrogenase activity is puzzling given that it is more than two orders of magnitude higher than the circulating level of free cortisol (1–100nM). While the hepatic oxo-reductase activity in homogenates proved unstable, the cloned rat liver  $11\beta$ -HSD1 enzyme behaves predominantly as a reductase in transfected toad bladder mucosa cells (Duperrex *et al.*, 1993) and Cos-1 cells (Low *et al.*, 1994a). Studies on human  $11\beta$ -HSD1 transiently or stably expressed in mammalian cells show that the enzyme has a higher affinity for cortisone (approximate  $K_m$  270nM) than for cortisol ( $K_m$  1.8 $\mu$ M) (Moore *et al.*, 1993; Stewart *et al.*, 1994) and are consistent with the finding that in cultured omental adipose stromal tissue, where there is abundant expression of message for  $11\beta$ -HSD1, the predominant reaction is the conversion of cortisone to cortisol (Bujalska *et al.*, 1997b). Similarly reductase activity is 5- to 10-fold higher than dehydrogenase activity in human skin fibroblasts (Hammami and Siiteri, 1991) and in rat aortic minces (Brem *et al.*, 1995). Functionally,  $11\beta$ -HSD1 by reactivating glucocorticoid has been shown to be of physiological relevance in cultured hippocampal cells (Rajan *et al.*, 1996), adipose stromal cells (Bujalska *et al.*, 1997b), gonad, epidermis and liver. In the liver, clinical studies have inhibited hepatic  $11\beta$ -HSD1 activity with carbenoxolone; the fall in hepatic glucose output has been attributed to a reduction in intrahepatic glucocorticoid levels (Walker *et al.*, 1995). This together with the emerging data on the putative role of  $11\beta$ -HSD1 in patients with visceral obesity has generated some interest in this enzyme as a modulator of insulin sensitivity. Elsewhere the enzyme is expressed in the luteinized theca cells of the ovary, Leydig cells of the testis, lung and decidua (Ricketts *et al.*, 1998).  $11\beta$ -HSD1 is expressed in proximal tubules within the rodent kidney, but is present at very low to undetectable levels in human and sheep kidneys (Yang *et al.*, 1992; Nikkila *et al.*, 1993; Whorwood *et al.*, 1995).

Western blot analysis revealed that a 34kD protein was highly expressed in rat liver, testis, kidney and lung, while a 26 kD species was also found in the brain (Monder and Lakshmi, 1990). Northern blot analysis showed a 1.7kb message in most tissues except the kidney where alternate promoter usage gave rise to additional multiple shorter species and evidence for a protein with a truncated N-terminal domain (Krozowski *et al.*, 1990; Krozowski *et al.*, 1992; Moisan *et al.*, 1992a, b). However, expression of the truncated enzyme did not produce a soluble protein (Mercer *et al.*, 1993). Further studies have also revealed a third putative  $11\beta$ -HSD1 congener in the sheep arising as the result of the deletion of exon 5 (Yang *et al.*, 1995). The three proteins are now referred to as  $11\beta$ -HSD1A,  $11\beta$ -HSD1B and  $11\beta$ -HSD1C, respectively, but there is currently no evidence that either  $11\beta$ -HSD1B or  $11\beta$ -HSD1C are active (Mercer *et al.*, 1993; Yang *et al.*, 1995). However, the association of  $11\beta$ -HSD1A with carbonyl reductase activity in mouse liver suggests that it may act on substrates including xenobiotics (Oppermann *et al.*, 1995; Maser *et al.*, 1996).

Examination of the  $11\beta$ -HSD1 peptide sequence revealed the presence of two potential N-linked glycosylation sites in the cloned rat enzyme (asparagine-X-serine, residues 158–160 and 203–205) consistent with the original description of the purified rat hepatic  $11\beta$ -HSD1 as a glycoprotein (Lakshmi and Monder, 1988). Mutagenesis studies show that modification of the first site decreased dehydrogenase and reductase activities to 75% and 50% of the wild type, while mutation of the second site caused an almost complete abolition of both activities (1995a).



### 11 $\beta$ -hydroxysteroid dehydrogenase type 2

11 $\beta$ -HSD1 immunoreactivity is apparently absent in the distal nephron, but elegant microdissection studies on the rabbit nephron indicated enzyme activity within the distal nephron and collecting ducts (Bonvalet *et al.*, 1990). Similar studies supported the presence of an additional NAD-dependent isozyme in rat kidney, subsequently called type 2 11 $\beta$ -HSD (11 $\beta$ -HSD2) (Naray-Fejes-Toth *et al.*, 1991; Walker *et al.*, 1992; Mercer and Krozowski, 1992). 11 $\beta$ -HSD2 has nearly 100 times the affinity for corticosterone and cortisol as 11 $\beta$ -HSD1 (Km for B, 10nM; for F, 40nM) and the reaction is unidirectional (Rusvai and Naray-Fejes-Toth, 1993; Brown *et al.*, 1993; Steward *et al.*, 1994).

The 11 $\beta$ -HSD2 enzyme was cloned from human (Albiston *et al.*, 1994) and sheep (Agarwal *et al.*, 1994) kidney using expression cloning in mammalian cells or *Xenopus* oocytes. The human enzyme is 405 residues in length and has a calculated molecular weight of 44, 140 daltons, although the purified protein and cloned species migrate at 40–41kDa on SDS gels (Krozowski *et al.*, 1995; Brown *et al.*, 1996a). Like 11 $\beta$ -HSD1 the 11 $\beta$ -HSD2 enzyme has a hydrophobic N-terminal domain that is thought to anchor the protein into the membrane. Truncation of this region does not, however, lead to solubilization of the enzyme and, unlike 11 $\beta$ -HSD1, deletion of the N-terminal domain does not abolish enzymatic activity. 11 $\beta$ -HSD2 has been cloned in other species including the rat (Zhou *et al.*, 1995), mouse (Cole 1995; Condon *et al.*, 1997) and rabbit (Naray-Fejes-Toth and Fejes-Toth, 1995).

There is evidence to support a nuclear localization for 11 $\beta$ -HSD2, although there is no classical nuclear localization signal in the protein. Confocal microscopy has demonstrated nuclear and cytoplasmic enzyme in human cortical collecting ducts and colon (Bujalska *et al.*, 1997a; Shimojo *et al.*, 1997), and western blot studies also show evidence of a nuclear protein. In the endometrium there appear to be two populations of cells: those in which the immunostaining is both cytoplasmic and nuclear, and cells with solely nuclear staining (Smith *et al.*, 1997). In other tissues, notably the placenta, no evidence for nuclear localization of 11 $\beta$ -HSD2 could be found (Petrelli *et al.*, 1997). Perinuclear localization of 11 $\beta$ -HSD2 has been shown using a fusion protein system (Naray-Fejes-Toth and Fejes-Toth, 1996), though this was interpreted by the authors as indicative of an endoplasmic reticulum localization. There is one potential N-glycosylation site (Asn<sup>394</sup>-Leu<sup>395</sup>-Ser<sup>396</sup>) in human 11 $\beta$ -HSD2, but when this site is mutated *in vitro*, or cells expressing 11 $\beta$ -HSD2 are treated with the glycosylation inhibitor tunicamycin, there is no loss of activity (Kynosse and Reeves, 1997).

11 $\beta$ -HSD2 is principally expressed in mineralocorticoid target tissues, kidney, colon and salivary gland, in keeping with its known physiological role in protecting the mineralocorticoid receptors (MR)(see later). Within these tissues it is expressed in the sodium-transporting epithelial cells. Expression is also high in the placenta and a number of fetal tissues (Brown *et al.* 1996b; Condon *et al.*, 1998) where its role is uncertain but may be involved in mediating fetal growth.

The characteristics of the human 11 $\beta$ -HSD isozymes are summarized in Table 12.1.

Table 12.1 Characteristics of human 11 $\beta$ -HSD isozymes.

	<i>Type 1</i>	<i>Type 2</i>
Enzyme kinetics	Low-affinity dehydrogenase, Km for F 2.1 $\mu$ M, Oxo-reductase, Km for E 0.3 $\mu$ M. NADP(H)-dependent	High affinity dehydrogenase, Km for F 50 nM. NAD-dependent
Principal sites distal of expression	Liver, lung, gonad, pituitary, cerebellum	Kidney (collecting ducts), colon, salivary gland, placenta
Molecular biology	1386 bp cDNA Encoding 292 aa protein	1873 bp cDNA Encoding 405 aa protein
Function <i>in vivo</i>	Predominately oxo-reductase Facilitation of glucocorticoid (GC) hormone action	Unidirectional dehydrogenase MR specificity
Pathophysiology	Possibly dysregulation in PCOS; Infertility; liver disease; GC feedback on HPA axis	Mutated HSD11B2 explains AME; inhibited by liquorice; Saturated in ectopic ACTH Syndrome; role in essential hypertension; placental 11 $\beta$ -HSD2, fetal development and adult hypertension

## GENETIC DEFECTS OF THE 11 $\beta$ -HSD ISOZYMES

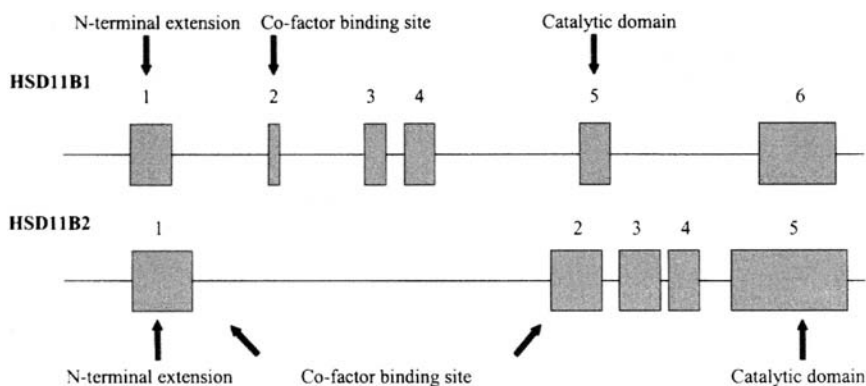
### A. 11 $\beta$ -HSD1

The human 11 $\beta$ -HSD1 gene encodes a protein of 292 amino acids. Exon 1 encodes the N-terminal extension, exon 2 encodes the cofactor binding domain, and exon 5 encodes the catalytic domain (White *et al.*, 1997). For a diagrammatic representation of the HSD11B1 gene see [Figure 12.1](#).

#### *Apparent cortisone reductase deficiency*

Seven patients with a defect in the peripheral conversion of E to F, have been reported (Taylor *et al.*, 1984, 1990; Phillipou and Higgins, 1985; Savage *et al.*, 1991; Nikkila *et al.* 1993; Phillipov *et al.*, 1996; Jamieson *et al.*, 1998). Six of the seven patients have been female and these have invariably presented in adolescence or early adulthood with features of hyperandrogenism (acne, hirsutism, oligo-amenorrhoea, infertility). Obesity has been a feature of some cases.

Serum androgens (testosterone, androstenedione and dehydroepiandrosterone sulfate) have been elevated in each case, but readily suppress following dexamethasone administration. Studies indicate an increased excretion of total cortisol metabolites indicative of enhanced cortisol secretion rates, often to values reported in patients with Cushing's syndrome. However, virtually all the urinary metabolites are excreted as 11-oxo-metabolites (THE, THA) with very low/undetectable levels of cortisol metabolites, (THF



**Figure 12.1** The structure of HSD11B1 and HSD11B2. The boxes represent the exons; those exons of functional importance are indicated.

and allo-THF) appearing in the urine. Typical THF+allo-THF/THE ratios, therefore, of <0.05 (normal adult range 0.7–1.3) have been reported. These data, together with an attenuated plasma cortisol response following oral cortisone acetate, suggest defective 11-oxo-reductase activity (and thus 11 $\beta$ -HSD1 activity). The defect in E to F conversion results in an increased metabolic clearance rate for cortisol; through the negative feedback mechanism ACTH secretion is increased to maintain normal circulating cortisol concentrations, but at the expense of ACTH-mediated androgen excess. Dexamethasone, by suppressing endogenous ACTH drive has been used therapeutically to control the hyperandrogenism. To date, the human 11 $\beta$ -HSD1 gene has been sequenced in only one patient with apparent cortisone reductase deficiency and this was the brother of a phenotypically affected female (Nikkilä *et al.*, 1993). 11 $\beta$ -HSD1 is the obvious candidate gene to explain apparent cortisone reductase deficiency, but to date no mutations have been reported; sequencing studies are thus required in other affected cases to elucidate the genetic cause. Disordered glycosylation of 11 $\beta$ -HSD1 may be a further possibility.

On the basis of these observations, two groups have analyzed cortisol metabolites in patients presenting with the commoner phenotype of polycystic ovary syndrome (PCOS). Both studies revealed an increase in cortisol secretion rates in the PCOS patients, and in one study a decrease in the THF+allo-THF/THE ratio was observed suggesting defective E to F conversion (Stewart *et al.*, 1990; Rodin *et al.*, 1994). Again this has not been evaluated at the genetic level.

#### *Recombinant mice lacking the HSD11B1 gene*

To further understand the importance of the 11 $\beta$ -HSD1 enzyme, mice have been generated bearing targeted disruption of the HSD11B1 gene. The HSD11B1 homozygous mutation was generated by removing the genomic fragment encompassing exons three and four through specific recombination in mouse embryonic stem cells. These mice were unable to convert inert 11-dehydrocorticosterone (cortisone in humans) to corticosterone (cortisol in

humans) *in vivo*. This led to attenuated activation of key hepatic enzymes (glucose-6-phosphatase and phosphoenolpyruvate) on starvation because of relative intrahepatic glucocorticoid deficiency (Kotelevtsev *et al.*, 1997). As a result knock-out mice did not become hyperglycaemic following stress or over-feeding and this occurred despite compensatory adrenal hyperplasia and an increased corticosterone secretion rate.

## B. 11 $\beta$ -HSD type2

The gene encoding 11 $\beta$ -HSD2 (HSD11B2) is located on the long arm of human chromosome 16 (16q22). The gene was isolated by screening a human genomic DNA library with a partial sheep 11 $\beta$ -HSD type 2 cDNA sequence. HSD11B2 consists of five exons spanning approximately 6.2Kb. Unlike HSD11B1, exon 1 of HSD11B2 lies approximately 3.5Kb upstream of the other exons. Exons one and two encode the putative NAD<sup>+</sup> cofactor binding site, and the putative catalytic binding site is encoded by exon four. The proximal promoter region of the gene has a high GC content and lacks TATA and CAAT boxes. There are putative binding sites for transcription factors including Spl and AP-2 (Agarwal *et al.*, 1995b). The encoded protein contains 405 amino acid residues and shares <15% sequence homology with the 11 $\beta$ -HSD1 gene; it has greater homology with 17 $\beta$ -HSD type 2 gene (37%) another member of the short chain dehydrogenase superfamily (Wu *et al.*, 1993). For a diagrammatic representation of the HSD11B2 gene see [Figure 12.1](#).

### *Apparent mineralocorticoid excess*

AME is a cause of low-renin, low-aldosterone hypertension and hypokalaemia found predominantly in children (Monder *et al.*, 1986; Dimartino-Nardi *et al.*, 1987; Stewart *et al.*, 1988; Milford *et al.*, 1995; Kitanaka *et al.*, 1996; White *et al.*, 1997). Children present with failure to thrive (low birth weight is a recognized feature), short stature, and have severe and often fatal hypertension and hypokalaemia. The profound hypokalaemia may cause rhabdomyolysis and nephrogenic diabetes insipidus manifesting as thirst and polyuria. Other renal abnormalities include renal cysts and nephrocalcinosis. Several cases with affected siblings have been reported and the condition is inherited as an autosomal recessive condition.

Defective peripheral conversion of cortisol to cortisone reflecting impaired activity of 11 $\beta$ -HSD was first suggested by Ulick, New and co-workers in patients with AME in the late 1970s (Ulick *et al.*, 1979) and has been further investigated by other groups. Urinary steroid metabolite profiles on such patients indicate that the majority of cortisol metabolites are excreted as THF and allo-THF with very low or absent levels of THE in the urine. The excretion of 5 $\alpha$ -cortisol metabolites exceeds that of 5 $\beta$ -cortisol metabolites resulting in a high urinary allo-THF/THF ratio suggesting an additional defect in 5 $\beta$ -reductase activity (Monder *et al.*, 1986). The incremental increase in the THF+allo-THF/THE compared to the allo-THF/THF ratio, however, is much larger, with typical THF+allo-THF/THE ratios ranging from 8 to greater than 70 in AME. The plasma half-life of [11 $\alpha$ -3H]-cortisol (which when metabolized by 11 $\beta$ -HSD yields tritiated water and cortisone), may more accurately

reflect renal  $11\beta$ -HSD2 activity (Ulick *et al.*, 1979; Stewart *et al.*, 1988), as may the ratio of urinary free cortisol/urinary free cortisone (UFF/UFE) (Palermo *et al.*, 1996; Best and Walker, 1997). Normal subjects excrete 2–3-fold more UFE than UFF, reflecting the expression of  $11\beta$ -HSD2 within the human kidney. In AME, however, UFE excretion is virtually undetectable (Palermo *et al.*, 1996). The conversion of cortisone to cortisol is normal in AME (Ulick *et al.*, 1979; Stewart *et al.*, 1988), all of which results in a marked increase in the plasma cortisol half-life. Despite this defect in the conversion of F to E, patients with AME are not Cushingoid; due to a normal intact negative feedback mechanism, cortisol secretion rate falls often to very low levels which maintain normal circulating concentrations in the face of impaired cortisol metabolism.

However, despite these normal circulating cortisol concentrations, cortisol was shown to have profound effects in the kidney and colon in AME patients by acting as a potent mineralocorticoid (Oberfield *et al.*, 1983; Dimartino-Nardi *et al.*, 1987; Stewart *et al.*, 1988). Thus an infusion of only 10mg/day hydrocortisone was shown to lower the urinary Na/K ratio, to suppress plasma renin activity and to increase measurements of subtraction potential difference (a marker of mineralocorticoid activity) across the rectal colon. Dexamethasone administration, by suppressing endogenous cortisol secretion, resulted in a natriuresis, potassium retention and lowered blood pressure with restoration of a normal renin-angiotensin-aldosterone system (Stewart *et al.*, 1988).

A second "variant" of AME, so-called "type II AME" has been documented. Type II AME has been described in 3 Sardinian patients and a further two cases from mainland Italy (Ulick *et al.*, 1990; Ulick *et al.*, 1992). This variant is characterized by a milder phenotype, with onset in late adolescence or early adulthood and by a relatively normal urinary THF+allo-THF/THE ratio. A generalized defect in cortisol A-ring metabolism was proposed as the underlying defect. However, the UFF/UFE excretion is high in the type II variant, and the metabolism of  $11\alpha$ -triated cortisol (directly reflecting  $11\beta$ -HSD activity) is grossly deranged, suggesting deficiency of  $11\beta$ -HSD2 (Mantero *et al.*, 1996). Evidence suggests that cortisol is also the offending mineralocorticoid in this "type II" variant (Tedde *et al.*, 1992). An acquired form of AME results following the ingestion of large quantities of liquorice and its metabolites (glycyrrhetic acid and carbenoxolone). The mineralocorticoid excess state results because these derivatives are potent inhibitors of  $11\beta$ -HSD. These unusual forms of hypertension have been discussed in detail elsewhere and are beyond the scope of this chapter (Stewart and Krozowski, 1999).

#### *Mineralocorticoid receptor specificity*

These clinical studies, however, provided crucial information surrounding the function of corticosteroid receptors (GR and MR). Paradoxically, the glucocorticoids, corticosterone and cortisol, have much higher affinity for the MR (Kd, 0.5–1nM) than for the GR (Kd, 10nM). Indeed *in vitro* binding studies on the partially purified hippocampal MR (Krozowski and Funder, 1983) and the expressed human MR cDNA (Arriza *et al.*, 1987) indicate that the MR has the same intrinsic affinity for aldosterone, cortisol and corticosterone. The much higher circulating concentrations of cortisol and corticosterone compared to aldosterone raises the question as to how aldosterone occupies the MR *in vivo*, and why the MR is not

swamped by glucocorticoid? Observations from patients with AME and from subjects consuming large quantities of glycyrrhetic acid, the active component of liquorice (Stewart *et al.*, 1987) indicate that it is  $11\beta$ -HSD2 which serves this role. Thus in normal physiology,  $11\beta$ -HSD2 inactivates F to E within epithelial cells of the renal collecting ducts and colonic mucosa, thereby preventing occupancy of the MR by F itself (Edwards *et al.*, 1988; Funder *et al.*, 1988). Aldosterone gains access to the MR and normal *in vivo* specificity is maintained. Animal data would support this hypothesis; inhibition of  $11\beta$ -HSD with the licorice derivatives glycyrrhetic acid and carbenoxolone confers mineralocorticoid potency upon physiological concentrations of endogenous glucocorticoids in both the rat kidney (Souness and Morris, 1989) and colon (Hierholzer *et al.*, 1990). Similar studies have been reported in a mineralocorticoid-responsive toad bladder cell line (Gaeggeler *et al.*, 1989).

#### *Genetic basis for AME*

It is now known that mutations in the gene encoding  $11\beta$ -HSD2 cause the syndrome of "apparent mineralocorticoid excess" (Wilson *et al.*, 1995a; Mune *et al.*, 1995; Stewart *et al.*, 1996). Because  $11\beta$ -HSD type 1 was the first  $11\beta$ -HSD enzyme to be characterized and cloned, AME patients were initially screened for mutations in their HSD11B1 gene to determine if mutations in this gene were responsible for the disease. No mutations were found in the four unrelated AME patients studied (Nikkila *et al.*, 1993).

AME type I has an autosomal recessive mode of inheritance, and is most commonly found in consanguineous families (Wilson *et al.*, 1995; Mune *et al.*, 1995; Stewart *et al.*, 1996). To date, 17 mutations have been reported in the HSD11B2 gene in 27 AME patients (Table 12.2) (White *et al.*, 1997).

Fifteen of the mutations are homozygous. The majority of the mutations identified result in a total loss of  $11\beta$ -HSD2 activity: they are R186C, R337H $\Delta$ 3nt, E356 $\Delta$ 1nt (causes reading frame shift) (Wilson *et al.*, 1995b; Ferrari *et al.*, 1996b), D224N and L250R, N286 $\Delta$ 1nt (causes reading frame shift) (Dave-Sharma *et al.*, 1998), L250P, L251S (Mune *et al.*, 1995; Ferrari *et al.*, 1996b), and A328V (Li *et al.*, 1997).

In a case under our care, a HSD11B2 mutation was found to result in a premature stop codon in exon 5 (R374X, a nonsense mutation) also resulting in a total loss of  $11\beta$ -HSD2 activity in an Asian boy (Stewart *et al.*, 1996). A younger brother was also affected and both parents were shown to be heterozygous for the mutation. A twin brother had died aged 3 and was presumably also affected and stillbirth was a further feature of this kindred. Indeed when placental tissue was obtained from this "stillbirth" pregnancy and the R374X mutation was confirmed, severe attenuation of placental  $11\beta$ -HSD2 activity (representing a fetal tissue) could be demonstrated. Subsequently the same mutation was identified by our group in a Dutch female who had been reported several years earlier (Harrinck *et al.*, 1984). An intronic mutation which also results in a loss of activity has been identified, a C to T transition mutation in intron 3 causes skipping of the fourth exon during processing of the pre-mRNA, and thus also causes a loss of activity (Mune *et al.*, 1995). The mutations L250P and L251S lie close to the catalytic domain and may alter the secondary structure from an  $\alpha$ -helix to a turn in this region and also make it less hydrophobic (Mune *et al.*, 1995). Mutations in exon 3 P227L, and exon 5 R337C cause a reduction in enzyme specificity

Table 12.2 11 $\beta$ -HSD2 mutations.

<i>Mutation</i>	<i>% Activity or <math>K_m</math></i>	<i>Reference</i>
R186C	0%	Wilson <i>et al.</i> , 1995b Ferrari <i>et al.</i> , 1996
R208C	1.5%	Mune <i>et al.</i> , 1995 Wilson <i>et al.</i> , 1995b Dave-Sharma <i>et al.</i> , 1998
R208H and R337H $\Delta$ Y338	0%	Kitanaka <i>et al.</i> , 1997
R213C	3.6%	Mune <i>et al.</i> , 1995
	0%	Rogoff <i>et al.</i> , 1998
P227L	$K_m = 284$ nM	Wilson <i>et al.</i> , 1997
Y232 $\Delta$ 9nt and G305 $\Delta$ 11nt	0%	Mune <i>et al.</i> , 1995
D244N and L250R	0%	Dave-Sharma <i>et al.</i> , 1998
L250P, L251S	0%	Mune <i>et al.</i> , 1995 Wilson <i>et al.</i> , 1995b Ferrari <i>et al.</i> , 1996c
N286 $\Delta$ 1nt	0%	Dave-Sharma <i>et al.</i> , 1998
A328V	0%	Li <i>et al.</i> , 1997
R337C	$K_m = 1010$ nM	Wilson <i>et al.</i> , 1995a Ferrari <i>et al.</i> , 1996a Obeyesekere, V.R., 1995
R337H $\Delta$ 3nt	0%	Wilson <i>et al.</i> , 1995b; Mune <i>et al.</i> , 1995
E356 $\Delta$ 1nt	0%	Wilson <i>et al.</i> , 1995b; Ferrari, P., 1996c
R374X	0%	Stewart <i>et al.</i> , 1996; Harnick <i>et al.</i> , 1984
Intron 3C to T	0%	Mune <i>et al.</i> , 1995
R279C	$K_m = 54$ nM, $V_{max} = 60\%$ wt	Li <i>et al.</i> , 1998

(Wilson *et al.*, 1995a, 1997; Ferrari *et al.*, 1996a). The mutations R208C and R213C lie in a region of 11 $\beta$ HSD2 which is homologous to the probable steroid binding domain of 3 $\alpha$ ,20 $\beta$ -HSD, and therefore these mutations may affect affinity of the enzyme for steroids (Ghosh *et al.*, 1991; Mune *et al.*, 1995). Thus, R208C and R213C do not affect steroid substrate binding in a direct fashion (Mune and White, 1996; White *et al.*, 1997; Rogoff *et al.*, 1998).

Only two of the AME patients are compound heterozygotes with both alleles coding for an enzyme devoid of activity, one (Irish/American) has the mutations Y232 $\Delta$ 9nt (which deletes a crucial catalytic residue) and G305 $\Delta$ 11nt (which causes a reading frame shift) (Mune *et al.*, 1995) and the other (Japanese) has the mutations R208H and R337H $\Delta$ Y338 (Kitanaka *et al.*, 1997). These two compound heterozygous patients were clinically indistinguishable from all other AME patients with homozygous HSD11B2 mutations.

*The evolution of the mutations*

The R337H $\Delta$ Y338 mutation has been found in three families and the Japanese compound heterozygote. The three families have a common ancestral background. One is of Zoroastrian descent from Iran, this religious group emigrated to the Bombay area in the seventh century, where the other two families live. This common ancestry supports a founder effect for this mutation (Wilson *et al.*, 1995b; Mune *et al.*, 1995). The Japanese mutation is believed to have arisen independently (Kitanaka *et al.*, 1997). Six AME kindreds are of native American origin, three of which carry the same mutation (L250S, L251P) which is also consistent with a founder effect. The others are all homozygous for a different mutation. A possible explanation for this is that heterozygosity for HSD11B2 mutations confer a selective advantage. It is possible that such heterozygotes have an increased ability to conserve sodium under conditions of severe sodium deprivation, these conditions may occur in inland America (White *et al.*, 1997). Heterozygotes are not clinically affected, although evidence from *in vitro* studies has shown that the activity and stability of some heterodimeric 11 $\beta$ HSD2 complexes may be compromised (Ferrari *et al.*, 1996a). This has been supported by modeling data which suggests that some mutations tend to destabilize dimer formation (Tsigelny and Baker, 1995). In some AME kindreds the heterozygote parents had evidence of mineralocorticoid based hypertension but presentation did not occur until late middle age (Stewart *et al.*, 1988; Li *et al.*, 1997). To fully understand the significance of the heterozygote state, prolonged follow-up of the parents with children affected with AME is required.

Nine of the seventeen HSD11B2 mutations involve amino acid residues encoding Arginine, and a number of these have originated independently. It has been suggested that this is due to the mutational rate of CpG dinucleotides. This is consistent with methylation-induced deamination of 5-methyl cytosine, and suggests that the HSD11B2 gene may be subject to methylation (Cooper and Youssoufian, 1988).

*AME "type II"*

A study of a Sardinian kindred with the type II variant of AME revealed a novel mutation, R279C, in the HSD11B2 gene. This mutation results in only mild attenuation of enzyme activity, encoding an enzyme with a normal  $K_m$  for cortisol, but with a  $V_{max}$  that was reduced by 33% of wild-type activity. It is possible that this mutation results in an altered affinity for the cofactor  $NAD^+$ , or that the amino acid substitution causes a structural change in the protein, decreasing its activity. It has been suggested that rather than dividing AME into two variants, the syndrome should be seen as a continuum, explained on the basis of mutations in the human HSD11B2 gene that have varying degrees of severity (Li *et al.*, 1998). In keeping with this suggestion, a recent case of mild low-renin hypertension was described in a patient from an inbred Mennonite family. Clinical studies showed a moderately elevated cortisol to cortisone metabolite ratio. The conversion of F to E *in vivo* was 58%, compared to 0–6% in AME patients whereas normal conversion is 90–95%. The molecular analysis of her HSD11B2 gene revealed a homozygous C to T transition in the second nucleotide of codon 227 resulting in a Pro to Leu substitution (P227L)(Wilson *et al.*,



1997). This supports the theory of the continuum of AME based on the spectrum of HSD11B2.

#### *AME genotype/phenotype correlations*

The biochemical phenotype of AME is best characterized by the urinary THE+allo-THF/ THE ratio. This value varies considerably between patients. It has been suggested that by using the THE+allo-THF/ THE ratio a correlation can be drawn between the activity of the expressed 11 $\beta$ -HSD2 mutant and biochemical severity (Mune and White, 1996). A more recent study, however, has shown difficulty in correlating genotype with the disease phenotype (Dave-Sharma *et al.*, 1998). These studies should be viewed cautiously, until a larger numbers of cases of AME patients with milder phenotypes can be assessed.

#### *Recombinant mice lacking the HSD11B2 gene*

Mice have been generated with targeted disruption of their 11 $\beta$ -HSD2 gene (Kotelevtsev *et al.*, 1999). The homozygous mutation was generated by removing the genomic fragment encompassing exons 2–5 through specific recombination in mouse embryonic stem cells. Fifty percent of the homozygous mutant mice died within 48 hours of birth, the remaining survivors were fertile and exhibited hypokalemia, hypotonic polyuria, and apparent mineralocorticoid activity of corticosterone. The young adult mice were markedly hypertensive. The epithelium of the distal tubule of the nephron displayed hypertrophy and hyperplasia. The homozygous mutant mice, therefore, showed the major features of AME and will be a useful model to evaluate the renal consequences of mineralocorticoid hypertension (Kotelevtsev *et al.*, 1999).

#### *HSD11B2 as a candidate gene for hypertension and renal disease*

The HSD11B2 gene has been suggested as a candidate gene for essential hypertension, as a compromise in 11 $\beta$ -HSD activity can result in severe hypertension. Hypertension is a multifactorial disorder with both genetic and environmental determinants, which affects 15–25% of the population. In 95% of these cases no underlying cause for the hypertension can be found, and these patients are labeled as having essential hypertension. Two previous studies have suggested impaired 11 $\beta$ -HSD activity in patients with hypertension (Walker *et al.*, 1993; Soro *et al.*, 1995), to investigate the role of 11 $\beta$ -HSD2 in essential hypertension, microsatellite markers have been employed.

Microsatellite markers are highly polymorphic dinucleotide repeats that are spread throughout the human genome. Due to these two properties they are useful tools for identifying disease associations. Two such microsatellites, D16S301 and D16S496, flank the HSD11B2 gene and have been used to investigate the genetic association between the gene encoding 11 $\beta$ -HSD2 and essential hypertension. The study analyzed the two microsatellite repeats in 79 unrelated black hypertensives with end stage renal disease and 168 black control subjects (Watson *et al.*, 1996). The study showed no association with the D16S301 marker, however, a significant association was found with the 216bp repeat allele of the

D16S496 microsatellite which was increased in the patients compared to the control subjects ( $p=0.008$ ). This result infers that a gene in close proximity to D16S496 is associated with essential hypertension; HSD11B2 lies proximal to this marker.

An investigation of the 11 $\beta$ -HSD2 gene in essential hypertension has also been performed in French Caucasians. Analysis of the gene using SSCP revealed a silent transition mutation (purine to purine) at codon position 534 in exon 3 (G534A). The frequency of this variant was determined in hypertensive patients ( $n=1153$ ) and control subjects ( $n=313$ ); however, no association was observed between the mutation and essential hypertension. The study also characterized a CA microsatellite repeat in the 30Kb cosmid clone containing HSD11B2. The frequency of the microsatellite repeat alleles was then analyzed in 347 siblings from 162 families. No association was found between any microsatellite allele and essential hypertension (Brand *et al.*, 1998).

A further study has identified a silent mutation (GAG to GAA) in exon 3 position 178 in Swiss subjects using a PCR-RFLP technique. The polymorphism was subsequently analyzed in normal control subjects ( $n=116$ ), end-stage renal disease ( $n=107$ ), diabetes mellitus ( $n=25$ ), and essential hypertension patients ( $n=41$ ). A correlation with the marker was observed between end stage renal transplant patients ( $n=61$ ) and normal controls ( $p=0.035$ ) (Smolenicka *et al.*, 1998). As this is a silent mutation it is unlikely that it is directly causing the association with end stage renal disease, but that it is in linkage disequilibrium with the true disease determining factor.

Due to the conflicting nature of these studies, further investigations need to be undertaken to elucidate the true role of HSD11B2 in essential hypertension.

### 17-HYDROXYSTEROID DEHYDROGENASES (17 $\beta$ -HSD)

Closely related members of the short-chain alcohol dehydrogenase (SCAD) superfamily, 17 $\beta$ -HSD catalyze the interconversion of less potent androgens and estrogens, androstenedione and estrone, with the more potent ligands, testosterone and estradiol respectively. Although seven 17 $\beta$ -HSD isozymes have been described, to date only types 1–4 have been well characterized in human tissues (Labrie *et al.*, 1997). A clinical phenotype has only been ascribed to mutations in the type 3 17 $\beta$ -HSD isozyme which results in a form of sexual hermaphroditism. This is discussed in more detail in [Chapter 11](#). Each isozyme demonstrates specific patterns of tissue distribution, oxidative or reductive activity, substrate specificity and co-factor dependency ([Table 12.3](#)) and, on this basis it is exciting to speculate the clinical phenotype of mutations in the genes encoding these isozymes. 17 $\beta$ -HSD type 7, also known as the Ke6 protein has been closely linked to the development of an autosomal recessive form of polycystic kidney disease in the mouse (Fomitcheva *et al.*, 1998). Deficiency of 17 $\beta$ -HSD type 4 leads to the disease known as Zellweger syndrome (results from the impairment of cerebro-hepato-renal peroxisomal functions) (Novikov *et al.*, 1997). Therefore, it could be postulated that mutations in the gene encoding this isozyme could be responsible for the disease.

**Table 12.3** 17 $\beta$ -HSD isozymes reported to date in mammalian species.

<i>Isozyme</i>	<i>Preferred substrate</i>	<i>Cofactor</i>	<i>Tissue expression</i>	<i>Comments</i>
17 $\beta$ -HSD1 (human)	estrone (E <sub>1</sub> )	NADPH	Placenta, ovary, breast, prostate, adipose	
17 $\beta$ -HSD2 (human)	estradiol (E <sub>2</sub> ) and testosterone	NAD	placenta, liver, gut, endometrium	Also has 20 $\alpha$ -HSD activity 30% homology to 11 $\beta$ -HSD2
17 $\beta$ -HSD3 (human)	androstenedione	NADPH	Testis	Genetic mutations result in male pseudohermaphroditism
17 $\beta$ -HSD4 (pig, human)	estradiol	NAD	Liver, prostate, gonad, gut, placenta, breast, kidney	Sterol carrier domain
17 $\beta$ -HSD5 (human)	androstenedione	NADPH	placenta, liver, muscle	
17 $\beta$ -HSD6 (rat)	3 $\alpha$ -diol	NAD	Prostate, kidney, liver	
17 $\beta$ -HSD7 (mouse)	estradiol (testosterone)	NAD	Kidney, gonad, liver	Also called the Ke6 protein

## REFERENCES

- Agarwal, A.K., Monder, C., Eckstein, B. and White, P.C. (1989) Cloning and expression of rat cDNA encoding corticosteroid 11 $\beta$ -dehydrogenase. *J. Biol. Chem.* **264**, 18939–18946.
- Agarwal, A.K., Mune, T., Monder, C. and White, P.C. (1994) NAD(+)-dependent isoform of 11 $\beta$ -hydroxysteroid dehydrogenase—Cloning and characterization of cDNA from sheep kidney. *J. Biol. Chem.* **269**, 25959–25962.
- Agarwal, A.K., Mune, T., Monder, C. and White, P.C. (1995a) Mutations in putative glycosylation sites of rat 11 $\beta$ -hydroxysteroid dehydrogenase affect enzymatic activity. *Biochim. Biophys. Acta* **1248**, 70–74.
- Agarwal, A.K., Rogerson, P.M., Mune, T. and White, P.C. (1995b) Gene structure and chromosomal localization of the human HSD11k gene encoding the kidney (type 2) isozyme of 11 $\beta$ -hydroxysteroid dehydrogenase. *Genomics* **29**, 195–199.
- Albiston, A.L., Obeyesekere, V.R., Smith, R.E. and Krozowski, Z.S. (1994) Cloning and tissue distribution of the human 11 $\beta$ -hydroxysteroid dehydrogenase type 2 enzyme. *Mol. Cell. Endocrinol.* **105**, R11–R17.
- Arriza, J.L., Simerly, R.B., Swanson, L.W. and Evans, R.M. (1988) The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. *Neuron* **1**, 887–900.
- Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Houseman, D.E., *et al.* (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* **237**, 268–275.
- Best, R. and Walker, B.R. (1997) Additional value of measurement of urinary cortisone and unconjugated cortisol metabolites in assessing the activity of 11 $\beta$ -hydroxysteroid dehydrogenase *in vivo*. *Clin. Endocrinol.* **47**, 231–236.

- Bonvalet, J.P., Doignon, I., Blot-Chabaud, M., Pradelles, P. and Farman, N. (1990) Distribution of 11 $\beta$ -hydroxysteroid dehydrogenase along the rabbit nephron. *J. Clin. Invest.* **86**, 832–837.
- Brem, A.S., Bina, R.B., King, T. and Morris, D.J. (1995) Bidirectional activity of 11 $\beta$ -hydroxysteroid dehydrogenase in vascular smooth muscle cells. *Steroids* **60**, 406–410.
- Brown, R.W., Chapman, K.E., Edwards, C.R.W. and Seckl, J.R. (1993) Human placental 11 $\beta$ -hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* **132**, 2614–2621.
- Brown, R.W., Chapman, K.E., Murad, P., Edwards, C.R.W. and Seckl, J.R. (1996a) Purification of 1 ip-hydroxysteroid dehydrogenase type 2 from human placenta utilizing a novel affinity labelling technique. *Biochem. J.* **313**, 997–1005.
- Brown, R.W., Diaz, R., Robson, A., Kotelevtsev, Y.V., Mullins, J.J., Kaufman, M.H. and Seckl, J.R. (1996b) The ontogeny of 11 $\beta$ -hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology* **137**, 794–797.
- Bujalska, I., Shimojo, M., Howie, A. and Stewart, P.M. (1997a) Human 11 $\beta$ -hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue. *Steroids* **77**, 77–82.
- Bujalska, I., Kumar, S. and Stewart, P.M. (1997b) Does central obesity reflect “Cushing’s disease of the omentum”? *Lancet* **349**, 1210–1213.
- Cole, T.J. (1995) Cloning of the mouse 11 $\beta$ -hydroxysteroid dehydrogenase type 2 gene: Tissue specific expression and localization in distal convoluted tubules and collecting ducts of the kidney. *Endocrinology* **136**, 4693–4696.
- Condon, J.C., Gosden, C., Gardener, D., Nixon, P., Hewison, M. and Stewart, P.M. (1998) Expression of type 2 11 $\beta$ -hydroxysteroid dehydrogenase and corticosteroid hormone receptors in early human fetal life. *J. Endocrinol.* **156S**, P282.
- Condon, J., Ricketts, M.L., Whorwood, C.B. and Stewart, P.M. (1997) Ontogeny and sexual dimorphic expression of mouse type 2 11 $\beta$ -hydroxysteroid dehydrogenase. *Mol. Cell. Endocrinol.* **127**, 121–128.
- Cooper, D.N. and Youssoufian, H. (1988) The CpG dinucleotide and human genetic disease. *Hum. Genet.* **78**, 151–155.
- Dave-Sharma, S., Wilson, R.C., Harbison, M.D., Newfield, R., Razzahy-Azar, M., Krozowski, Z.S., *et al.* (1998) Examination of genotype and phenotype relationships in 14 patients with apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **83**, 2244–2254.
- Dimartino-Nardi, J., Stoner, E., Martin, K., Balfe, J.W., Jose, P.A and New, M.I. (1987) New findings in apparent mineralocorticoid excess. *Clin. Endocrinol.* **27**, 49–62.
- Duperrex, H., Kenouch, S., Gaeggeler, H.P., Seckl, J.R., Edwards, C., Farman, N. and Rossier, B.C. (1993) Rat liver 11 $\beta$ -hydroxysteroid dehydrogenase complementary deoxyribonucleic acid encodes oxoreductase activity in a mineralocorticoid-responsive toad bladder cell line. *Endocrinology* **132**, 612–619.
- Edwards, C.R.W., Stewart, P.M., Burt, D., Brett, L., McIntyre, M.A., Sutanto, W.S., *et al.* (1988) Localisation of 11 $\beta$ -hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* **ii**, 986–989.
- Ferrari, P., Obeyesekere, V.R., Li, K., Andrews, R.K. and Krozowski, Z.S. (1996a) The 11 $\beta$ -hydroxysteroid dehydrogenase type II enzyme: biochemical consequences of the congenital R337C mutation. *Steroids* **61**, 197–200.
- Ferrari, P., Obeyesekere, V.R., Li, K., Wilson, R.C., New, M.I., Funder, J.W., *et al.* (1996b). Point mutations abolish 11 $\beta$ -hydroxysteroid dehydrogenase type II activity in three families with the congenital syndrome of apparent mineralocorticoid excess. *Mol. Cell. Endocrinol.* **119**, 21–24.

- Fomitcheva, J., Baker, M.E., Anderson, E., Lee, G.Y. and Aziz, N. (1998) Characterisation of Ke6, a new 17 $\beta$ -hydroxysteroid dehydrogenase, and its expression in gonadal tissues. *J. Biol. Chem.* **273** (35), 22664–22671.
- Funder, J.W., Pearce, P.T., Smith, R. and Smith, A.I. (1988) Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242**, 583–585.
- Gaeggeler, H.P., Edwards, C.R. and Rossier, B.C. (1989) Steroid metabolism determines mineralocorticoid specificity in the toad bladder. *Am. J. Physiol.* **257**, F690–F695.
- Hammami, M.M. and Siiteri, P.K. (1991) Regulation of 11 $\beta$ -hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action. *J. Clin. Endocrinol. Metab.* **73**, 326–334.
- Harinck, H.I.J., Van Brummelen, P., Van Steers, A.P. and Moolenaar, A.J. (1984) Apparent mineralocorticoid excess and deficient 11 $\beta$ -oxidation of cortisol in a young female. *Clin. Endocrinol.* **21**, 505–514.
- Hierholzer, K., Siebe, H. and Fromm, M. (1990) Inhibition of 11 $\beta$ -hydroxysteroid dehydrogenase and its effect on epithelial sodium transport. *Kidney Int.* **38**, 673–678.
- Jamieson, A., Wallace, A.M., Walker, B.R., Fraser, R. and Connell, J.M.C (1998) Apparent cortisone reductase deficiency: clinical and biochemical features. *J. Endocrinol.* **156S**, P65.
- Jenkins, J.S. (1966) The metabolism of cortisol by human extrahepatic tissues. *J. Endocrinol.* **34**, 51–56.
- Kitanaka, S., Katsumata, N., Tanae, A., Hibi, I., Takeyama, K.I., Fuse, H., *et al.* (1997) A new compound heterozygous mutation in the 11 $\beta$ -hydroxysteroid dehydrogenase type 2 gene in a case of apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **82**, 4054–4058.
- Kitanaka, S., Tanae, A. and Hibi, I. (1996) Apparent mineralocorticoid excess due to 11 $\beta$ -hydroxysteroid dehydrogenase deficiency: a possible cause of intrauterine growth retardation. *Clin. Endocrinol.* **44**, 353–359.
- Kotelevtsev, Y., Brown, R., Fleming, S., Kenyon, C., Edwards, C.R.W., Seckl, J.R., *et al.* (1999) Hypertension in mice lacking 11 $\beta$ -hydroxysteroid dehydrogenase type 2. *J. Clin. Invest.* **103**, 683–689.
- Kotelevtsev, Y., Holmes, M.C., Burchell, A., Houston, P.M., Schmoll, D., Jamieson, P., *et al.* (1997) 11 $\beta$ -hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14924–14929.
- Krozowski, Z. (1992) 11 $\beta$ -Hydroxysteroid dehydrogenase and the short-chain alcohol dehydrogenase (SCAD) superfamily. *Mol. Cell. Endocrinol.* **84**, C25–C31.
- Krozowski, K. and Funder, J.W. (1983) Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6056–6060.
- Krozowski, Z., Maguire, J.A., Stein-Oakley, A.N., Dowling, J., Smith, R.E. and Andrews, R.K. (1995) Immunohistochemical localization of the 11 $\beta$ -hydroxysteroid dehydrogenase type II enzyme in human kidney and placenta. *J. Clin. Endocrinol. Metab.* **80**, 2203–2209.
- Krozowski, Z., Obeyesekere, V., Smith, R. and Mercer, W. (1992) Tissue-specific expression of an 11 $\beta$ -hydroxysteroid dehydrogenase with a truncated N-terminal domain. A potential mechanism for differential intracellular localization within mineralocorticoid target cells. *J. Biol. Chem.* **267**, 2569–2574.
- Krozowski, Z., Stuchbery, S., White, P., Monder, C. and Funder, J.W. (1990). Characterization of 11 $\beta$ -hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology* **127**, 3009–3013.

- Kyosseff, Z.N. and Reeves, W.B. (1997) N-glycosylation is not essential for enzyme activity of 11 $\beta$ -hydroxysteroid dehydrogenase type 2. *Kidney Int.* **52**, 682–686.
- Labrie, F., Luu-The, V., Lin, S.-X., Labrie, C., Simard, J., Breton, R., *et al.* (1997) The key role of 17 $\beta$ -hydroxysteroid dehydrogenase in sex steroid biology. *Steroids* **62**, 148–158.
- Lakshmi, V. and Monder, C. (1988) Purification and characterization of the corticosteroid 11 $\beta$ -dehydrogenase component of the rat liver 11 $\beta$ -hydroxysteroid dehydrogenase complex. *Endocrinology* **123**, 2390–2398.
- Li, A.R., Li, K.X.Z., Marui, S., Krozowski, Z.S., Batista, M.C., Whorwood, C.B., *et al.* (1997) Apparent mineralocorticoid excess in a Brazilian kindred: hypertension in the heterozygote state. *J. Hypertension* **15**, 1397–1402.
- Li, A., Tedde, R., Krozowski, Z.S., Pala, A., Li, K.X.Z., Shackleton, C.H.L., *et al.* (1998) Molecular basis for hypertension in the “type II variant” of apparent mineralocorticoid excess. *Am. J. Hum. Genet.* **63**, 370–379.
- Low, S.C., Assaad, S.N., Rajan, V., Chapman, K.E., Edwards, C.R.W. and Seckl, J.R. (1993) Regulation of 11 $\beta$ -hydroxysteroid dehydrogenase by sex steroids *in vivo*: further evidence for the existence of a second dehydrogenase in rat kidney. *J. Endocrinol.* **139**, 27–35.
- Mantero, F., Palermo, M., Petrelli, M.D., Tedde, R., Stewart, P.M. and Shackleton, C.H.L. (1996) Apparent mineralocorticoid excess: Type I and type II. *Steroids* **61**, 193–196.
- Maser, E., Richter, E. and Frieberthaus, J. (1996) The identification of 11 $\beta$ -hydroxysteroid dehydrogenase as carbonyl reductase of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Eur. J. Biochem.* **238**, 484–489.
- Mercer, W.R. and Krozowski, Z.S. (1992) Localization of an 11 $\beta$ -hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinology* **130**, 540–543.
- Mercer, W., Obeyesekere, V., Smith, R. and Krozowski, Z. (1993) Characterization of 11 $\beta$ HSD1B gene expression and enzymatic activity. *Mol. Cell. Endocrinol.* **92**, 247–251.
- Milford, D.V., Shackleton, C.H.L. and Stewart, P.M. (1995) Mineralocorticoid hypertension and congenital deficiency of 11 $\beta$ -hydroxysteroid dehydrogenase in a family with the syndrome of “apparent” mineralocorticoid excess. *Clin. Endocrinol.* **43**, 242–246.
- Moisan, M.P., Edwards, C. and Seckl, J.R. (1992a) Differential promoter usage by the rat 11 $\beta$ -hydroxysteroid dehydrogenase gene. *Mol. Endocrinol.* **6**, 1082–1087.
- Moisan, M.P., Edwards, C.R. and Seckl, J.R. (1992b) Ontogeny of 11 $\beta$ -hydroxysteroid dehydrogenase in rat brain and kidney. *Endocrinology* **130**, 400–404.
- Monder, C. and Lakshmi, V. (1989) Evidence for kinetically distinct forms of corticosteroid 11 $\beta$ -dehydrogenase in rat liver microsomes. *J. Steroid Biochem.* **32**, 77–83.
- Monder, C. and Lakshmi, V. (1990) Corticosteroid 11 $\beta$ -dehydrogenase of rat tissues: Immunological studies. *Endocrinology* **126**, 2435–2443.
- Monder, C., Shackleton, C.H.L., Bradlow, H.L., New, M.I., Stoner, E., Iohan, F., *et al.* (1986) The syndrome of apparent mineralocorticoid excess: its association with 11 $\beta$ -dehydrogenase and 5 $\beta$ -reductase deficiency and some consequences for corticosteroid metabolism. *J. Clin. Endocrinol. Metab.* **63**, 550–557.
- Moore, C.D.C., Mellon, S.H., Murai, J., Siiteri, P.K. and Miller, W.L. (1993) Structure and function of the hepatic form of 11 $\beta$ -hydroxysteroid dehydrogenase in the squirrel monkey, an animal model of glucocorticoid resistance. *Endocrinology* **133**, 368–375.
- Mune, T., Rogerson, F.M., Nikkila, H., Agarwal, A.K. and White, P.C. (1995) Human hypertension caused by mutations in the kidney isozyme of 11 $\beta$ -hydroxysteroid dehydrogenase. *Nat. Genet.* **10**, 394–399.

- Mune, T. and White, P.C. (1996) Apparent mineralocorticoid excess: Genotype is correlated with biochemical phenotype. *Hypertension* **27**, 1193–1199.
- Naray-Fejes-Toth, A. and Fejes-Toth, G. (1995) Expression cloning of the aldosterone target cell-specific 11 $\beta$ -hydroxysteroid dehydrogenase from rabbit collecting duct cells. *Endocrinology* **136**, 2579–2586.
- Naray-Fejes-Toth, A. and Fejes-Toth, G. (1996) Subcellular localization of the type 2 11 $\beta$ -hydroxysteroid dehydrogenase—a green fluorescent protein study. *J. Biol. Chem.* **271**, 15436–15442.
- Naray-Fejes-Toth, A., Watlington, C.O. and Fejes-Toth, G. (1991) 11 $\beta$ -Hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* **129**, 17–21.
- Nikkila, H., Tannin, G.M., New, M.I., Taylor, N.F., Kalaitzoglou, G., Monder, C., *et al.* (1993) Defects in the HSD11 gene encoding 11 $\beta$ -hydroxysteroid dehydrogenase are not found in patients with apparent mineralocorticoid excess or 11-oxoreductase deficiency. *J. Clin. Endocrinol. Metab.* **77**, 687–691.
- Novikov, D., Dieuaide-Noubhani, M., Vermeesch, J.R., Fournier, B., Mannaerts, G.P. and Van Veldhoven, P.P. (1997) The human peroxisomal multifunctional protein involved in bile acid synthesis: activity measurement, deficiency in Zellweger syndrome. *Biochim. Biophys. Acta* **1360**, 229–240.
- Oberfield, S.E., Levine, L.S., Carey, R.M., Greig, F., Ullick, S. and New, M.I. (1983) Metabolic and blood pressure responses to hydrocortisone in the syndrome of apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **56**, 332–338.
- Oppermann, U., Netter, K.J. and Maser, E. (1995) Cloning and primary structure of murine beta-hydroxysteroid dehydrogenase microsomal carbonyl reductase. *Eur. J. Biochem.* **227**, 202–208.
- Osinski, P.A. (1960) Steroid 11 $\beta$ -ol dehydrogenase in human placenta. *Nature* **187**, 777.
- Ozols, J. (1995) Lumenal orientation and post-translational modifications of the liver microsomal 11 $\beta$ -hydroxysteroid dehydrogenase. *J. Biol. Chem.* **270**, 2305–2312.
- Palermo, M., Shackleton, C.H.L., Mantero, F. and Stewart, P.M. (1996) Urinary free cortisone and the assessment of 11 $\beta$ -hydroxysteroid dehydrogenase activity in man. *Clin. Endocrinol.* **45**, 605–611.
- Petrelli, M.D., Lim-Tio, S.S., Condon, J., Hewison, M. and Stewart, P.M. (1997) Differential expression of nuclear 11 $\beta$ -hydroxysteroid dehydrogenase type 2 in mineralocorticoid receptor positive and negative tissues. *Endocrinology* **138**, 3077–3080.
- Phillipou, G. and Higgins, B.A. (1985) A new defect in the peripheral conversion of cortisone to cortisol. *J. Steroid Biochem.* **22**, 435–436.
- Phillipov, G., Palermo, M. and Shackleton, C.H.L. (1996) Apparent cortisone reductase deficiency: a unique form of hypercortisolism. *J. Clin. Endocrinol. Metab.* **81**, 3855–3860.
- Rajan, V., Chapman, K.E., Lyons, V., Jamieson, P., Mullins, J.J., Edwards, C.R.W., *et al.* (1995) Cloning, sequencing and tissue distribution of mouse 11 $\beta$ -hydroxysteroid dehydrogenase-1 cDNA. *J. Steroid Biochem. Molec. Biol.* **52**, 141–147.
- Rajan, V., Edwards, C.R.W. and Seckl, J.R. (1996) 11 $\beta$ -hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. *J. Neuroscience* **16**, 65–70.
- Ricketts, M.L., Verhaeg, J.M., Bujalska, I., Howie, A.J., Rainey, W.E. and Stewart, P.M. (1998) Immunolocalization of type I 11 $\beta$ -hydroxysteroid dehydrogenase in human tissues. *J. Clin. Endocrinol. Metab.* **83**, 1325–1335.
- Rodin, A., Thakkar, H., Taylor, N. and Clayton, R. (1994) Hyperandrogenism in polycystic ovary syndrome: evidence of dysregulation of 11 $\beta$ -hydroxysteroid dehydrogenase. *N. Engl. J. Med.* **330**, 460–465.

- Rogoff, D., Smolenicka, Z., Bergada, I., Vallejo, G., Barontini, M., Heinrich, J.J. and Ferrari, P. (1998) The codon 213 of the of 11 $\beta$ -hydroxysteroid dehydrogenase type 2 gene is a hot spot for mutations in apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **83**, 4391–4393.
- Rusvai, E. and Naray-Fejes-Toth, A. (1993) A new isoform of 11 $\beta$ -hydroxysteroid dehydrogenase in aldosterone target cells. *J. Biol. Chem.* **268**, 10717–10720.
- Savage, M.W., Barton, R.N., Dornan, T.L., Horan, M.A., Robins, A.J. and Taylor, N.F. (1991) Increased metabolic clearance of cortisol in corticosteroid 11-reductase deficiency. *J. Endocrinol.* **129S**, 219.
- Shimojo, M., Ricketts, M.L., Petrelli, M.D., Moradi, P., Johnson, G.D., Bradwell, A.R., *et al.* (1997) Immunodetection of 11 $\beta$ -hydroxysteroid dehydrogenase type 2 in human mineralocorticoid target tissues: Evidence for nuclear localization. *Endocrinology* **138**, 1305–1311.
- Smith, R.E., Salamonsen, L.A., Komesaroff, P.A., Li, K.X., Myles, K.M., Lawrence, M., *et al.* (1997) 11 $\beta$ -Hydroxysteroid dehydrogenase type II in the human endometrium: localization and activity during the menstrual cycle. *J. Clin. Endocrinol. Metab.* **82**, 4252–4257.
- Smolenicka, Z., Bach, E., Schaer, A., Liechti-Gallati, S., Frey, B.M., Frey, F.J., *et al.* (1998) A new polymorphic restriction site in the human of 11 $\beta$ -hydroxysteroid dehydrogenase type 2 gene. *J. Clin. Endocrinol. Metab.* **83**, 1814–1817.
- Soro, A., Ingram, M.C., Tonolo, G., Glorioso, N. and Fraser, R. (1995) Evidence of coexisting changes in 11 $\beta$ -hydroxysteroid dehydrogenase and 5 $\beta$ -reductase activity in patients with untreated essential hypertension. *Hypertension* **25**, 67–70.
- Souness, G.W. and Morris, D.J. (1989) The antinatriuretic and kaluretic effects of the glucocorticoids corticosterone and cortisol following pre-treatment with carbenoxolone sodium (a liquorice derivative) in the adrenalectomized rat. *Endocrinology* **124**, 1588–1590.
- Stewart, P.M., Corrie, J.E.T., Shackleton, C.H.L. and Edwards, C.R.W. (1988) Syndrome of apparent mineralocorticoid excess: a defect in the cortisol—cortisone shuttle. *J. Clin. Invest.* **82**, 340–349.
- Stewart, P.M. and Krozowski, Z.S. (1999) 11 $\beta$ -hydroxysteroid dehydrogenase. *Vit. Horm.* **57**, 249–324.
- Stewart, P.M., Krozowski, Z.S., Gupta, A., Milford, D.V., Howie, A.J., Sheppard, M.C., *et al.* (1996) Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11 $\beta$ -hydroxysteroid dehydrogenase type 2 gene. *Lancet* **347**, 88–91.
- Stewart, P.M., Murry, B.A. and Mason, J.I. (1994) Human kidney 11 $\beta$ -hydroxysteroid dehydrogenase is a high affinity NAD<sup>+</sup>-dependent enzyme and differs from the cloned “type I” isoform. *J. Clin. Endocrinol. Metab.* **79**, 480–484.
- Stewart, P.M., Shackleton, C.H.L., Beastall, G.H. and Edwards, C.R.W. (1990) 5 $\alpha$ -reductase activity in poly cystic ovary syndrome. *Lancet* **335**, 431–433.
- Stewart, P.M., Wallace, A.M., Valentino, R., Burt, D., Shackleton, C.H.L. and Edwards, C.R.W. (1987) Mineralocorticoid activity of liquorice: 11 $\beta$ -hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* **ii**, 821–824.
- Tannin, G.M., Agarwal, A.K., Monder, C., New, M.I. and White, P.C. (1991) The human gene for 11 $\beta$ -hydroxysteroid dehydrogenase: structure, tissue distribution, and chromosomal localization. *J. Biol. Chem.* **266**, 16653–16658.
- Taylor, N.F., Bartlett, W.A. and Dawson, D.J. (1984) Cortisone reductase deficiency: evidence for a new inborn error in metabolism of adrenal steroids. *J. Endocrinol.* **102S**, 89.
- Taylor, N.F., Pollock, A. and Dornan, T.L. (1990) Corticosteroid 11-reductase deficiency: steroid studies in a further family. *J. Clin. Invest.* **13** (Suppl. 2), 238.



- Tedde, R., Pala, A., Melis, A. and Ulick, S. (1992) Evidence for cortisol as the mineralocorticoid in the syndrome of apparent mineralocorticoid excess. *J. Clin. Invest.* **15**, 471–474.
- Tsigelny, I. and Baker, M.E. (1995). Structures stabilizing the dimer interface on human 11 $\beta$ -hydroxysteroid dehydrogenase types 1 and 2 and human 15-hydroxyprostaglandin dehydrogenase and their homologs. *Biochem. Biophys. Res. Commun.* **217**, 859–868.
- Ulick, S., Levine, L.S., Gunczler, P., Zanconato, G., Ramirex, L.C., Rauh, W., *et al.* (1979) A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J. Clin. Endocrinol. Metab.* **49**, 757–764.
- Ulick, S., Tedde, R. and Mantero, F. (1990) Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **70**, 200–206.
- Ulick, S., Tedde, R. and Wang, J.Z. (1992) Defective ring a reduction of cortisol as the major metabolic error in the syndrome of apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **74**, 593–599.
- Walker, B.R., Campbell, J.C., Williams, B.C. and Edwards, C.R.W. (1992) Tissue-specific distribution of the NAD<sup>+</sup>-dependent isoform of 11 $\beta$ -hydroxysteroid dehydrogenase. *Endocrinology* **131**, 970–972.
- Walker, B.R., Connacher, A.A., Lindsay, R.M., Webb, D.J. and Edwards, C. (1995) Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. *J. Clin. Endocrinol. Metab.* **80**, 3155–3159.
- Walker, B.R., Stewart, P.M., Shackleton, C.H.L., Padfield, P.L. and Edwards, C.R.W. (1993) Deficient inactivation of cortisol by 11 $\beta$ -hydroxysteroid dehydrogenase in essential hypertension. *Clin. Endocrinol.* **39**, 221–227.
- Watson, B., Bergman, S.M., Myracle, A., Callen, D.F., Acton, R.T. and Warnock, D.G. (1996) Genetic association of 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (HSD11B2) flanking microsatellites with essential hypertension in blacks. *Hypertension* **28**, 478–482.
- White, P.C., Mune, T. and Agarwal, A.K. (1997) 11 $\beta$ -hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. *Endocr. Rev.* **18**, 135–156.
- Whorwood, C.B., Mason, J.I., Ricketts, M.L., Howie, A.J. and Stewart, P.M. (1995) Detection of human 11 $\beta$ -hydroxysteroid dehydrogenase isoforms using reverse transcriptase-polymerase chain reaction and localization of the type 2 isoform to renal collecting ducts. *Mol. Cell. Endocrinol.* **110**, R7–R12.
- Wilson, R.C., Dave-Sharma, S., Wei, J.Q., Obeyesekere, V.R., Li, K., Ferrari, P., *et al.* (1998) A genetic defect resulting in mild low-renin hypertension. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10200–10205.
- Wilson, R.C., Krozowski, Z.S., Li, K., Obeyesekere, V.R., Razzaghyazar, M., Harbison, M.D., *et al.* (1995a) A mutation in the HSD11B2 gene in a family with apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **80**, 2263–2266.
- Wilson, R.C., Harbison, M.D., Krozowski, Z.S., Funder, J.W., Shackleton, C., Hanauskeabel, H.M., *et al.* (1995b) Several homozygous mutations in the gene for 11 $\beta$ -hydroxysteroid dehydrogenase type 2 in patients with apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **80**, 3145–3150.
- Wu, L., Einstein, M., Geissler, W.M., Chan, H.K., Elliston, K.O. and Andersson, S. (1993) Expression cloning and characterization of human 17 $\beta$ -hydroxysteroid dehydrogenase type 2, a microsomal enzyme possessing 20 $\alpha$ -hydroxysteroid dehydrogenase activity. *J. Biol. Chem.* **268**, 12964–12969.
- Yang, K., Smith, C.L., Dales, D., Hammond, G.L. and Challis, J.R.G. (1992) Cloning of an ovine 11 $\beta$ -hydroxysteroid dehydrogenase complementary deoxyribonucleic acid—tissue and temporal

- distribution of its messenger ribonucleic acid during fetal and neonatal development. *Endocrinology* **131**, 2120–2126.
- Yang, K., Yu, M. and Han, V.K.M. (1995) Identification and tissue distribution of a novel variant of 11-hydroxysteroid dehydrogenase 1 transcript. *J. Steroid Biochem. Molec. Biol.* **55**, 247–253.
- Zhou, M.Y., Gomez-Sanchez, E.P., Cox, D.L., Cosby, D. and Gomez-Sanchez, C.E. (1995) Cloning, expression, and tissue distribution of the rat nicotinamide adenine dinucleotide-dependent 11 $\beta$ -hydroxysteroid dehydrogenase. *Endocrinology* **136**, 3729–3734.

# 13.

## ANIMAL MODELS OF IMPAIRED STEROIDOGENESIS

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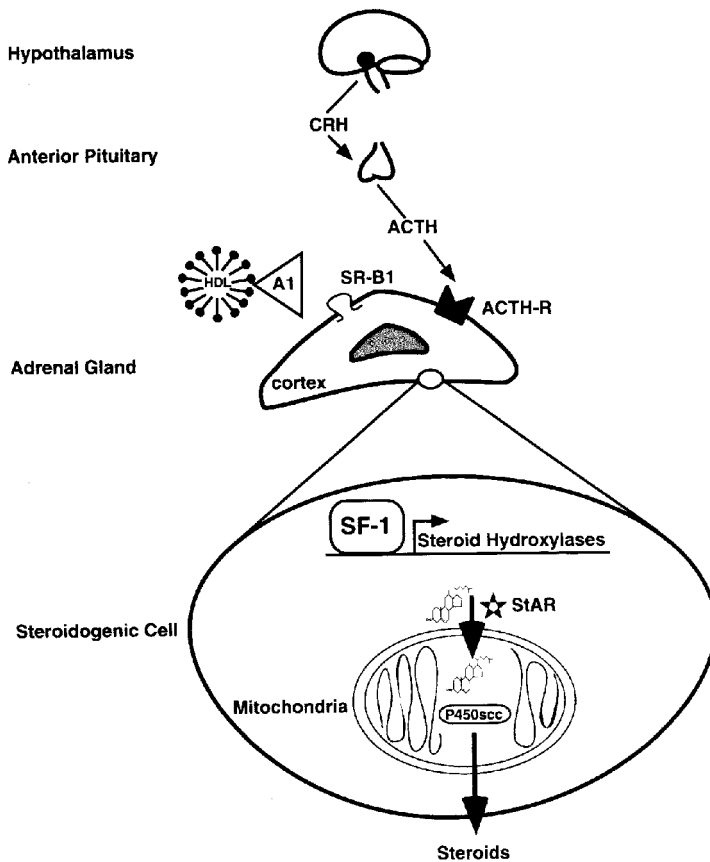
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Animal models of impaired steroidogenesis provide a valuable complement to clinical studies of human patients, permitting experimental manipulations that are not possible in humans and facilitating the use of novel methodologies such as targeted gene disruption and transgenic rescue to create new genetic models. As outlined below, these animal models encompass the full spectrum of steroidogenesis—ranging from defects at the hypothalamic-pituitary levels that indirectly affect steroid production to defects in circulating lipoproteins that affect the delivery of substrate for steroidogenesis to intrinsic defects within the steroidogenic tissues that impair steroidogenesis. In this chapter, we provide an overview of the currently available animal models, focusing particularly on abnormalities intrinsic to the steroidogenic organs. These animal models provide opportunities to explore the potential of gene therapy, possibly leading to the development of methods for the genetic correction of human inborn errors of steroidogenesis.

KEY WORDS: genetics, gene therapy, knockout mice.

### INTRODUCTION

As detailed elsewhere in this volume, the regulated production of steroid hormones requires complex, reciprocal interactions among the hypothalamus, anterior pituitary, and primary steroidogenic tissues. As a consequence of this complexity, defects at multiple levels can impair steroidogenesis. [Figure 13.1](#) presents an overview of the multiple factors that control the appropriate production of steroid hormones, using the adrenal cortex as a model. Analyses of human patients with clinical disorders resulting from impaired steroidogenesis have provided key insights into the roles of a number of these components (see [Chapters 5–12](#)). These human studies—coupled with cell culture analyses in steroidogenic cells from different species—provide a framework for understanding the essential steps in steroid production. There remain, however, experiments that cannot be performed in humans. In addition, evolving technologies for making transgenic and knockout mice have expanded enormously our ability to examine *in vivo* the effects of mutating different components of the steroidogenic complex. This chapter discusses the various animal models of impaired steroidogenesis, both those arising naturally and those resulting from genetic manipulation.



**Figure 13.1 Schematic overview of the multiple steps in steroidogenesis.** A diagram of the different levels involved in the regulation of steroid hormone production is shown. CRH, corticotropin-releasing factor; ACTH, corticotropin; ACTH-R, ACTH receptor; HDL, high density lipoprotein; A1, apolipoprotein A-1; SR-B1, scavenger receptor-B1; SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein; P450scc, cholesterol side-chain cleavage enzyme.

As detailed in [Table 13.1](#) and discussed below, it is useful conceptually to divide these disorders into those that result from intrinsic defects in the steroidogenic cells (i.e., the primary defects) and those that are secondary to defects at other levels.

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**Table 13.1** Animal models of impaired steroidogenesis.

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<i>Primary defects</i>
Defects in cholesterol delivery to the steroidogenic pathway
Watanabe LDL receptor deficient rabbit (Hoeg <i>et al.</i> , 1985)
SiAR knockout mouse (Caron <i>et al.</i> , 1997)
Defects in steroidogenic enzymes
P450scc deficient rabbit (Yang <i>et al.</i> , 1993)
C4 deficient mouse with 21-hydroxylase deficiency (Shiroishi <i>et al.</i> , 1987)
Aromatase knockout mouse (Fisher <i>et al.</i> , 1998; Honda <i>et al.</i> , 1998)
Defects in the development of the steroidogenic organs
SF-1 knockout mouse (Luo <i>et al.</i> , 1994)
WT1 knockout mouse (Kreidberg <i>et al.</i> , 1993)
Adrenocortical dysplasia mouse (Beamer <i>et al.</i> , 1994)
Lim1 knockout mouse (Shawlot and Behringer, 1995)
Emx2 knockout mouse (Miyamoto <i>et al.</i> , 1997)
M33 knockout mouse (Katoh-Fukui <i>et al.</i> , 1998)
<i>Secondary defects</i>
Impaired cholesterol delivery to steroidogenic organs
Apolipoprotein A1 knockout mouse (Plump <i>et al.</i> , 1996)
Hypothalamic/pituitary abnormalities
CRH knockout mouse (Muglia <i>et al.</i> , 1995)
CRFR1 knockout mouse (Smith <i>et al.</i> , 1998; Timpl <i>et al.</i> , 1998)
hypogonadal mouse (Mason <i>et al.</i> , 1986a,b)
gonadotrope ablation mouse (Kendall <i>et al.</i> , 1991; Burrows <i>et al.</i> , 1996)
$\alpha$ -subunit of glycoprotein hormones knockout mouse (Kendall <i>et al.</i> , 1995)
Proopiomelanocortin ablation mouse (Allen <i>et al.</i> , 1995)

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## DEFECTS INTRINSIC TO STEROIDOGENIC CELLS

### Defects in cholesterol delivery to the steroidogenic pathway

#### *SR-B1 knockout mouse*

The scavenger receptor-B1, which is expressed at high levels in steroidogenic cells of the adrenal cortex and gonads, has been proposed to play important roles in the uptake of HDL cholesterol. Thus, one might predict that impaired steroidogenesis also would result from a deficiency of SR-B1 in species such as rodents where HDL serves as an important source of cholesterol substrate (Anderson and Dietschy, 1978). Consistent with this model, sequestration of SR-B1 with specific antibodies against its extracellular domain impairs steroidogenesis in mouse Y1 adrenocortical cells (Temel *et al.*, 1997). SR-B1 knockout mice have been generated (Rigotti *et al.*, 1997), and provide an opportunity to test *in vivo* the hypothesis that HDL cholesterol serves as an important source of cholesterol for steroidogenic cells.

*StAR knockout mouse*

As shown in Figure 13.1, an essential component of regulated steroidogenesis is the translocation of cholesterol from the cytoplasm to the inner mitochondrial membrane, where the cholesterol side-chain cleavage enzyme (P450<sub>scc</sub>, aka CYP11A) carries out the first committed step in steroidogenesis. Analyses of human patients with the inherited disorder of steroidogenesis, congenital lipoid adrenal hyperplasia (lipoid CAH), showed that a 30kDa mitochondrial phospho-protein—the steroidogenic acute regulatory protein (StAR)—is essential for this translocation (reviewed by Bose *et al.*, 1996). To explore the roles of StAR in a system amenable to experimental manipulation, a knockout mouse model of StAR deficiency was produced by targeted gene disruption (Caron *et al.*, 1997). At birth, StAR knockout mice are indistinguishable from wild-type littermates, except that males and females have female external genitalia. StAR knockout mice fail to grow normally thereafter and die within two weeks of birth from adrenocortical insufficiency. Hormone assays reveal severe defects in adrenal steroids, with loss of negative feedback regulation at hypothalamic-pituitary levels. In contrast, hormones constituting the gonadal axis do not differ significantly from levels in wild-type littermates. Histologically, the adrenal cortex of newborn StAR knockout mice contains striking lipid deposits, with lesser deposits in the steroidogenic compartment of the testis and none in the ovary. These sex-specific differences in gonadal pathology support a two-hit model of the pathogenesis of StAR deficiency (Bose *et al.*, 1996), in which trophic hormone stimulates progressive accumulation of lipids within the steroidogenic cells, ultimately causing the complete loss of steroidogenic competence as the cells die. These StAR knockout mice hopefully will provide a useful model system for determining the mechanisms that underlie StAR's essential roles in adrenocortical and gonadal steroidogenesis. Moreover, the potential to derive cell lines from the steroidogenic organs of these StAR knockout mice should provide an ideal setting to explore the structure-function aspects of StAR within the context of bona fide steroidogenic cells.

### Defects in steroidogenic enzymes

*Cholesterol side-chain cleavage enzyme (CYP11A) deficient rabbit*

The first animal model of impaired steroidogenesis to be described was a rabbit model of CYP11A deficiency (Fox and Crary, 1978). Because the gene encoding CYP11A is deleted (Pang *et al.*, 1992; Yang *et al.*, 1993), these rabbits are defective in the production of all classes of steroids, leading to adrenocortical insufficiency and male-to-female sex reversal of the external genitalia. Histologically, the adrenal glands exhibit marked hyperplasia, loss of normal zonation, and striking lipid deposits within the adrenocortical cells. Although these features resemble closely the histological findings in lipoid CAH in humans and in StAR knockout mice, it now is appreciated that these similar disorders result from mutations in two distinct genes: CYP11A and StAR. To date, no human patients have been identified with clinical disorders of steroidogenesis caused by mutations of CYP11A. Because placental steroids are essential to maintenance of human pregnancy (Casey and MacDonald, 1998), it

has been proposed that fetuses with homozygous deficiency of P450scc will die *in utero* (Miller, 1998).

#### *C4 deficient mouse with 21-hydroxylase deficiency*

The most common inborn error of steroidogenesis in humans results from a deficiency of 21-hydroxylase, which is encoded by a gene residing within the HLA major histocompatibility complex (Donohoue *et al.*, 1999). A mouse model of 21-hydroxylase deficiency—the H-2<sup>w18</sup> mouse—results from deletion of the region of mouse chromosome 17 that contains the 21-hydroxylase gene and the gene encoding the fourth component of serum complement (Shiroishi *et al.*, 1987; Gotoh *et al.*, 1988). As a result of this deletion, and the consequent lack of 21-hydroxylase activity, these mice cannot make glucocorticoids or mineralocorticoids and die of adrenocortical insufficiency within the first 2 weeks of life unless rescued by corticosteroid treatment. Unlike humans, the mouse adrenal cortex cannot produce androgens because it lacks steroid 17 $\alpha$ -hydroxylase; virilization of the female external genitalia therefore does not occur. These mice provided the first opportunity to demonstrate gene therapy of a primary steroidogenic defect, as a 21-hydroxylase transgene rescued the H-2<sup>w18</sup> mice from early postnatal lethality (Gotoh *et al.*, 1994).

#### *Aromatase knockout mouse*

Aromatase is the cytochrome P450 enzyme (CYP19) that catalyzes the conversion of C<sub>19</sub>-androgens to C<sub>18</sub>-estrogens. In human females with aromatase deficiency, the external genitalia are virilized at birth, consistent with the failure of placental aromatase to inactivate placental androgens by converting them to estrogens (Grumbach and Conte, 1998). At puberty, affected females do not experience breast development or menarche, but develop pubic and axillary hair. Their ovaries are polycystic. The one reported aromatase-deficient male underwent normal pubertal maturation and had macroorchidism. Subjects of both sexes have tall stature with delayed bone maturation and epiphyseal fusion and osteoporosis, consistent with an important role of estrogen in skeletal physiology. To explore the roles of aromatase *in vivo*, CYP19-knockout mice were generated (Fisher *et al.*, 1998; Honda *et al.*, 1998). Adult female mice exhibit sexual infantilism, with underdeveloped external genitalia, uteri, and mammary glands. Their ovaries contain follicles with increased numbers of granulosa cells, and stromal hyperplasia also is present. Follicular maturation is blocked before ovulation, resulting in abundant atretic follicles and absent corpora lutea. Male aromatase knockout mice have normal internal anatomy and normal testes, but the male accessory sex glands are enlarged due to an increased content of secreted material. Unlike the females, they are fertile and produce litters of normal size. Serum estradiol levels are considerably decreased, whereas testosterone, follicle-stimulating hormone, and luteinizing hormone are elevated. Although studies still are ongoing, the bones of male and female aromatase knockout mice exhibit histopathology consistent with the osteoporosis seen in human subjects (E. Simpson, personal communication). The aromatase knockout mice, in conjunction with estrogen receptor  $\alpha$  and P knockout mice, hopefully will provide novel insights into the mechanisms of estrogen action *in vivo*.

## Defects in the development of the steroidogenic organs

### *Steroidogenic factor 1 (SF-1) knockout mouse*

SF-1 was first identified as a transcription factor that regulates cell-specific expression of the cytochrome P450 steroid hydroxylases, which catalyze most of the reactions required for the synthesis of steroid hormones (Rice *et al.*, 1991; Morohashi *et al.*, 1992). The subsequent isolation of a cDNA encoding SF-1 established that this factor belongs to the nuclear hormone receptor family of transcription factors that mediate the actions of steroid hormones, thyroid hormone, vitamin D, and retinoids. In addition to the steroid hydroxylases, SF-1 also regulates adrenal and gonadal expression of many genes involved in steroidogenesis, including 3 $\beta$ -hydroxysteroid dehydrogenase, the ACTH receptor, StAR and SR-B1 (reviewed by Parker and Schimmer, 1997). Analyses of reporter genes driven by the Müllerian inhibiting substance (MIS) promoter region in transfected Sertoli cells and transgenic mice suggest that SF-1 also regulates expression of the MIS gene (Shen *et al.*, 1994; Hatano *et al.*, 1994; Guili *et al.*, 1997). Thus, it appears that SF-1 controls the production of both hormones that mediate male phenotypic differentiation: MIS and testosterone.

SF-1 is expressed in both male and female mouse embryos from the very earliest stages of gonadogenesis, when the intermediate mesoderm condenses to form the urogenital ridge (Ikeda *et al.*, 1994). As the testes differentiate, SF-1 expression localizes to both functional compartments: the interstitial region, where Leydig cells produce androgens, and the testicular cords, where Sertoli cells produce MIS. In contrast, SF-1 transcripts in ovaries decrease coincident with sexual differentiation, suggesting that persistent SF-1 expression may impair female sexual differentiation. In addition to the gonads and adrenals, SF-1 transcripts also are detected in the anterior pituitary and hypothalamus, suggesting that SF-1 regulates the endocrine axis at other levels.

Analyses of SF-1 knockout mice confirmed essential roles of SF-1 at all three levels of the hypothalamic-pituitary-steroidogenic organ axis. These SF-1 knockout mice lack adrenal glands and gonads, with the adrenal and gonadal anlage undergoing programmed cell death just as they normally undergo differentiation (Luo *et al.*, 1994; Sadovsky *et al.*, 1995). As a consequence of degeneration of testes before androgens and MIS are produced, SF-1 knockout mice exhibit male-to-female sex reversal of the internal and external urogenital tracts. These mice also have impaired expression of a number of markers of gonadotropes (Ingraham *et al.*, 1995; Shinoda *et al.*, 1995)—the cells in the anterior pituitary gland that regulate gonadal steroidogenesis—and they lack the ventromedial hypothalamic nucleus (Shinoda *et al.*, 1995; Ikeda *et al.*, 1995), a cell group in the medial hypothalamus linked to feeding and reproductive behaviors (Cantreras *et al.*, 1994).

Mutations in SF-1 in humans have not yet been demonstrated, and thus it has not been established that the roles defined in mice are relevant to human endocrine function. The human SF-1 gene shares extensive homology with its mouse counterpart (Oba *et al.*, 1996; Wong *et al.*, 1996) and is expressed in many of the same sites (Ramayya *et al.*, 1997), suggesting that SF-1 in humans functions much as it does in mice. Consistent with this model, analyses of the human SF-1 gene on chromosome 9q33 (Taketo *et al.*, 1996) have identified two



different SF-1 mutations associated with a clinical phenotype of adrenal insufficiency (Achermann *et al.*, 1999; Biason-Lauber *et al.*, 2000). Further studies are needed to define fully the spectrum of endocrine disease associated with SF-1 mutations in humans.

## SECONDARY DEFECTS IN STEROID HORMONE BIOSYNTHESIS

In addition to defects that directly impair the ability of steroidogenic cells to produce steroids, mutations that indirectly impair cholesterol delivery or that act at hypothalamic/pituitary levels also can prevent normal steroidogenesis. A summary of these animal models is provided in Table 13.1; some of the models are discussed briefly below.

In rodents, circulating HDL is the primary exogenous source of cholesterol for steroidogenic cells (Anderson and Dietschy, 1978). Apolipoprotein A-I (apoA-I), an important structural component of HDL, apparently plays an important role in SR-B1-mediated uptake of HDL cholesterol by steroidogenic cells. ApoA-I knockout mice have diminished basal levels of corticosteroid production, as well as a blunted steroidogenic response to stress (Plump *et al.*, 1996), supporting an important role for apoA-I in steroidogenesis. Of interest, male mice are affected to a greater degree than female mice, suggesting sexual dimorphism in the relative dependence of steroidogenesis on HDL-derived cholesterol. The relevance of these findings to steroidogenesis in humans remains to be defined, as humans probably obtain circulating cholesterol substrate most efficiently from LDL cholesterol.

Glucocorticoid production by the zonae fasciculata/reticularis of the adrenal cortex is regulated by ACTH, which in turn is regulated by CRH (see Figure 13.1). Analyses of CRH knockout mice revealed diminished corticosterone response to ACTH or stress, accompanied by marked atrophy of the zona fasciculata of the adrenal cortex (Muglia *et al.*, 1995). CRH knockout mice also fail to exhibit the normal circadian variation in plasma ACTH and corticosterone levels (Muglia *et al.*, 1997), suggesting that a normal diurnal increase in ACTH is essential to maintain normal adrenocortical function. Very similar findings are seen in knockout mice lacking one of the two subtypes of CRH receptor, CRFR1, which have low basal corticosterone levels associated with marked agenesis of the zona fasciculata of the adrenal cortex (Smith *et al.*, 1998; Timpl *et al.*, 1998).

## SUMMARY AND PERSPECTIVES

As outlined in this chapter, a diverse group of animal models are now available in which steroidogenesis is impaired due either to primary defects within steroidogenic cells or to secondary effects at other levels. In at least one case, the steroidogenic defect is inducible, such that normal actions of steroid hormones in development are maintained until pharmacological ablation of corticotropes expressing thymidine kinase by treatment with ganciclovir (Allen *et al.*, 1995). Such inducible ablation of cell lineages involved in steroidogenesis, coupled with inducible and tissue-specific knockouts (Lobe and Nagy, 1998), will provide a powerful means to manipulate different components of the steroidogenic complex and then analyze the effects *in vivo*. In addition, genetic manipulation via transgenesis already has been used to correct the steroidogenic defects in the H-2<sup>w18</sup> and

hypogonadal mice, an approach that holds considerable promise both for studying structure-function relationships of the steroidogenic enzymes and for modeling approaches to gene therapy of human disorders of steroidogenesis (Mason *et al.*, 1986b; Gotoh *et al.*, 1994). Thus, it appears certain that these animal models, in conjunction with knockout mice that lack the various steroid hormone receptors, will play an increasing role in extending our understanding of the complex processes of steroidogenesis and steroid hormone action.

## REFERENCES

- Achermann, J.C., Ito, M., Ito, M., Hindmarsh, P.C. and Jameson, J.L. (1999) A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. *Nat. Genet.* **22**, 125–126.
- Allen, R.G., Carey, C., Parker, J.D., Mortrud, M.T., Mellon, S.H. and Low, M.J. (1995) Targeted ablation of pituitary proopiomelanocortin cells by herpes simplex virus-1 thymidine kinase differentially regulates mRNAs encoding the adrenocorticotrophin receptor and aldosterone synthase in the mouse adrenal gland. *Mol. Endocrinol.* **9**, 1005–1016.
- Anderson, J.M. and Dietschy, J.M. (1978) Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary, and testis of the rat. *J. Biol. Chem.* **253**, 9024–9032.
- Beamer, W.G., Sweet, H.O., Bronson, R.T., Shire, J.G.M., Orth, D.N. and Davisson, M.T. (1994) Adrenocortical dysplasia: a mouse model system for adrenocortical insufficiency. *J. Endocrinol.* **141**, 33–43.
- Biason-Lauber, A. and Schoenle, E.J. (2000) Apparently normal ovarian differentiation in a prepubertal girl with transcriptionally inactive steroidogenic factor 1 (NR5A1/SF-1) and adrenocortical insufficiency. *Am. J. Hum. Genet.* **67**, 1563–1568.
- Bose, H.S., Sugawara, T., Strauss, III, J.F. and Miller, W.L. (1996) The pathophysiology and genetics of congenital lipid adrenal hyperplasia. *New Engl. J. Med.* **335**, 1870–1878.
- Canteras, N.S., Simerly, R.B. and Swanson, L.W. (1994) Organization of projections from the ventromedial hypothalamic nucleus of the hypothalamus: a *Phaseolus vulgaris*-leucoagglutinin study in the rat. *J. Comp. Neurol.* **348**, 41–79.
- Caron, K.M., Soo, S.-C., Clark, B.J., Stocco, D.M., Wetsel, W. and Parker, K.L. (1997) Targeted disruption of the mouse gene encoding the steroidogenic acute regulatory protein provides insights into congenital lipid adrenal hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11540–11545.
- Casey, L.M. and MacDonald, P.M. (1998) Endocrinology of pregnancy. In: *Williams Textbook of Endocrinology*, 9th Edition, Wilson, J.D., Foster, D.W., Kronenberg, H. and Larsen, P.R. (eds), Saunders, Philadelphia, pp. 1259–1266.
- Donohoue, P.A., Parker, K.L. and Migeon, C.J. (1995) Congenital adrenal hyperplasia. In: *The Metabolic and Molecular Bases of Inherited Disease*, Scriver, C.R., Beaudet, L.L., Sly, W.S. and Valle, D. (eds), McGraw-Hill, Inc., New York, pp. 2929–2966.
- Fisher, C.R., Graves, K.H., Parlow, A.F. and Simpson, E.R. (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the CYP19 gene. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6965–6970.
- Fox, R.R. and Crary, D.D. (1978) Genetics and pathology of hereditary adrenal hyperplasia in the rabbit. *J. Hered.* **69**, 251–254.
- Giuli, G., Shen, W.H. and Ingraham, H.A. (1997) The nuclear receptor SF-1 mediates sexually dimorphic expression of Mullerian Inhibiting Substance, *in vivo*. *Development* **124**, 1799–1807.

- Gotoh, H., Kusakabe, M., Shiroishi, T. and Moriwaki, K. (1994) Survival of steroid 21-hydroxylasedeficient mice without endogenous corticosteroids after neonatal treatment and genetic rescue by transgenesis as a model system for treatment of congenital adrenal hyperplasia in humans. *Endocrinology* **135**, 1470–1476.
- Gotoh, H., Sagai, T., Hata, J., Shiroishi, T. and Moriwaki, K. (1988) Steroid 21-hydroxylase deficiency in mice. *Endocrinology* **123**, 1923–1927.
- Grumbach, M.M. and Conte, F.A. (1998) Disorders of sexual differentiation. In: *Williams Textbook of Endocrinology*, 9th Edition, Wilson J.D., Foster, D.W., Kronenberg, H. and Larsen, P.R. (eds), Saunders, Philadelphia, pp. 1374–1375.
- Hatano, O., Takayama, K., Imai, T., Waterman, M.R., Takakusu, A., Omura, T., *et al.* (1994) Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. *Development* **120**, 2787–2797.
- Hoeg, J.M., Loriaux, L., Gregg, R.E., Green, W.R. and Brewer Jr. H.B. (1985) Impaired adrenal reserve in the Watanabe heritable hyperlipidemic rabbit: implications for LDL receptor function in steroidogenesis. *Metabolism* **34**, 194–197.
- Honda, S.-L., Harada, N., Ito, S., Takagi, Y. and Maeda, S. (1998) Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the CYP19 gene. *Biochem. Biophys. Res. Commun.* **252**, 445–449.
- Ikeda, Y., Luo, X., Abbud, R., Nilson, J.H. and Parker, K.L. (1995) The nuclear receptor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. *Mol. Endocrinol.* **9**, 478–486.
- Ikeda, Y., Shen, W.-H., Ingraham, H.A. and Parker, K.L. (1994) Developmental expression of mouse steroidogenic factor 1, an essential regulator of the steroid hydroxylases. *Mol. Endocrinol.* **8**, 654–662.
- Ingraham, H.A., Lala, D.S., Ikeda, Y., Luo, X., Shen, W.-H., Nachtigal, M.W., *et al.* (1994) The nuclear receptor SF-1 acts at multiple levels of the reproductive axis. *Genes Dev.* **8**, 2302–2312.
- Katoh-Fukui, Y., Tsuchiya, R., Shiroishi, T., Nakahara, Y., Hashimoto, N., Noguchi, K., *etal.* (1998) Male-to-female sex reversal in M33 mutant mice. *Nature* **393**, 688–692.
- Kendall, S.K., Samuelson, L.C., Saunders, T.L., Wood, R.I. and Camper, S.A. (1995) Targeted disruption of the pituitary glycoprotein hormone alpha-subunit produces hypogonadal and hypothyroid mice. *Genes Dev.* **9**, 2007–2019.
- Kendall, S.K., Saunders, T.L., Jin, L., Llod, R.V., Glode, L.M., Nett, T.M., *et al.* (1991) Targeted ablation of pituitary gonadotrophes in transgenic mice. *Mol. Endocrinol.* **5**, 2025–2036.
- Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., *et al.* (1993) WT-1 is required for early kidney development. *Cell* **74**, 679–691.
- Lobe, C.G. and Nagy A. (1998) Conditional genomic alteration in mice. *Bioessays* **20**, 200–208.
- Luo, X., Ikeda, Y. and Parker, K.L. (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell* **77**, 481–490.
- Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young, III, W.S., Phillips, H.S., Nikolics, K., *et al.* (1986a) A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science* **234**, 1366–1371.
- Mason, A.J., Pitts, S.L., Nikolics, K., Szonyi, E., Wilcox, J.N., Seeburg, P.H., *et al.* (1986b) The hypogonadal mouse: reproductive functions restored by gene therapy. *Science* **234**, 1372–1378.
- Miller, W.L. (1998) Why nobody has P450scc (20, 22-desmolase) deficiency. *J. Clin. Endocrinol. Metab.* **83**, 1399–1400.
- Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I. and Aizawa, S. (1997) Defects of urogenital development in mice lacking Emx2. *Development* **124**, 1653–1664.

- Morohashi, K., Honda, S., Inomata, Y., Handa, H. and Omura, T. (1992) A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *J. Biol. Chem.* **267**, 17913–17919.
- Muglia, L., Jacobson, L., Dikkes, P. and Majzoub, J.A. (1995) Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. *Nature* **373**, 427–432.
- Muglia, L.J., Jacobson, L., Weninger, S.C., Luedke, C.E., Bae, D.S., Jeong, K.H., *et al.* (1997) Impaired diurnal adrenal rhythmicity restored by constant infusion of corticotropin-releasing hormone in corticotropin-releasing hormone-deficient mice. *J. Clin. Invest.* **99**, 2923–2929.
- Oba, K., Yanase, T., Nomura, M., Morohashi, K., Takayanagi, R. and Nawata, H. (1996) Structural characterization of human Ad4BP (SF-1) gene. *Biochem. Biophys. Res. Commun.* **226**, 261–267.
- Pang, S., Yang, X., Wang, M., Tissot, R., Nino, M., Manaligod, J., *et al.* (1992) Inherited congenital adrenal hyperplasia in the rabbit: absent cholesterol side-chain cleavage cytochrome P450 gene expression. *Endocrinology* **131**, 181–186.
- Parker, K.L. and Schimmer, B.P. (1997) Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocr. Rev.* **18**, 361–377.
- Plump, A.S., Erickson, S.K., Weng, W., Partin, J.S., Breslow, J.L. and Williams, D.L. (1996) Apolipoprotein A-I is required for cholesteryl ester accumulation in Steroidogenic cells and for normal adrenal steroid production. *J. Clin. Invest.* **97**, 2660–2671.
- Ramayya, M.S., Zhou, J., Kino, T., Segars, J.H., Bondy, C.A. and Chrousos, G.P. (1997) Steroidogenic factor 1 messenger ribonucleic acid expression in Steroidogenic and nonsteroidogenic human tissues: Northern blot and *in situ* hybridization studies. *J. Clin. Endocrinol. Metab.* **82**, 1799–1806.
- Rice, D.A., Mouw, A.R., Bogerd, A. and Parker, K.L. (1991) A shared promoter element regulates the expression of three Steroidogenic enzymes. *Mol. Endocrinol.* **5**, 1552–1561.
- Rigotti, A., Trigatti, B.L., Penman, M., Rayburn, H., Herz, J. and Krieger, M. (1997) A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12610–12615.
- Sadovsky, Y., Crawford, P.A., Woodson, K.G., Polish, J.A., Clements, M.A., Tourtellotte, L.M., *et al.* (1995) Mice deficient in the orphan receptor Steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10939–10943.
- Shawlot, W. and Behringer, R.R. (1995) Requirement for Lim1 in head-organizer function. *Nature* **374**, 425–430.
- Shen, W.-H., Moore, C.C.D., Ikeda, Y., Parker, K.L. and Ingraham, H.A. (1994) Nuclear receptor Steroidogenic factor 1 regulates MIS gene expression: a link to the sex determination cascade. *Cell* **77**, 651–661.
- Shiroishi, T., Sagai, T., Natsume-Sakai, S. and Moriwaki, K. (1987) Lethal deletion of the complement component C4 and steroid 21-hydroxylase genes in the mouse H-2 class III region, caused by meiotic recombination. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2819–2823.
- Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., *et al.* (1998) Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* **20**, 1093–1102.
- Taketo, M., Parker, K.L., Howard, T.A., Tsukiyama, T., Wong, M., Niwa, O., *et al.* (1995) Homologs of Drosophila Fushi Tarazu Factor 1 map to mouse chromosome 2 and human chromosome 9q33. *Genomics* **25**, 565–567.

- Temel, R.E., Trigatti B., DeMattos, R.B., Azhar, S., Krieger M. and Williams, D.L. (1997) Scavenger receptor class B, type I (SR-BI) is the major route for delivery of high density lipoprotein cholesterol to the Steroidogenic pathway in cultured mouse adrenocortical cells. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13600–13605.
- Timpl, P., Spanagel, R., Sillaber, L., Kresse, A., Reul, J.M., Stalla, G.K., *et al.* (1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat. Genet.* **19**, 162–166.
- Wong, M., Ramayya, M.S., Chrousos, G.P., Driggers, P.H. and Parker, K.L. (1996) Cloning and sequence analysis of the human gene encoding Steroidogenic factor I. *J. Mol. Endocrinol.* **17**, 139–147.
- Yang, X., Iwamoto, K., Wang, M., Artwohl, J., Mason, J.I. and Pang, S. (1993) Inherited congenital adrenal hyperplasia in the rabbit is caused by a deletion in the gene encoding cytochrome P450 cholesterol side-chain cleavage enzyme. *Endocrinology* **132**, 1977–1982.

## 14.

# REGULATION OF GENE EXPRESSION BY THE NUCLEAR RECEPTOR FAMILY

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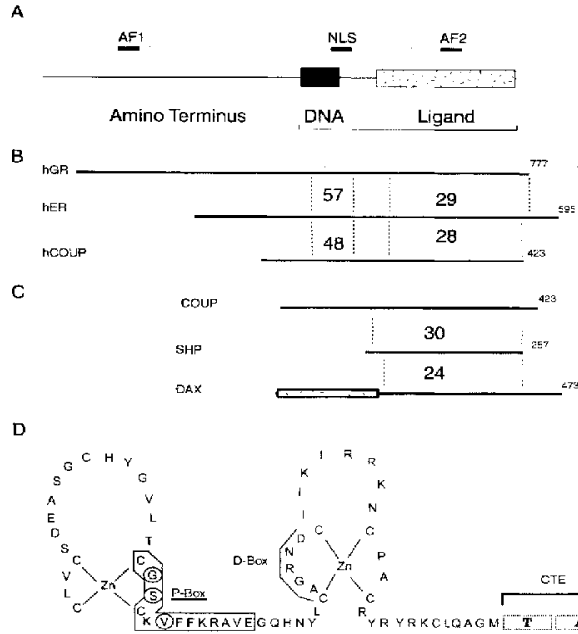
Nuclear receptors comprise a large family of transcription factors that regulate diverse biological processes, ranging from functions important in development and in homeostasis. The family contains both receptors for known ligands and a large number of “orphan” receptors for which ligands have not been identified.

Considerable insight has been gained in the last decade into the mechanisms by which nuclear receptors regulate the activities of responsive genes. Increasingly detailed structural information has become available that has illuminated the mechanisms by which specific DNA sequences are recognized by monomeric or dimeric receptor DBDs. In the case of the ligand-responsive members of this family, the determination of the structures of agonist- and antagonist-bound LBDs of several receptors has reinforced the conclusions derived from experiments focused on structural-functional relationships using the techniques of molecular biology. In addition, the identification of nuclear receptor coregulators and their roles in the formation and stabilization of the transcription initiation complex has permitted the elucidation of important functional links between the nuclear receptors and the general transcription apparatus of the cell.

KEYWORDS: Nuclear receptor, gene expression, coactivator, corepressor.

## INTRODUCTION

Prior to 1959, steroid hormones were proposed to exert their effects on target tissues via a variety of potential mechanisms, including the direct modification of the activity of “enzyme systems” or alteration of cell permeability (Szego and Roberts, 1953; Mueller, 1958). The initial insights into the mechanisms by which these effects were exerted in target cells came as a result of the capacity to prepare tritium-labeled steroids of sufficiently high specific activity to allow the detection of picogram quantities of steroids in tissue or cell preparations (Glasscock and Hoekstra, 1959; Jensen and Jacobson, 1960). These experiments, first conducted using tritiated estradiol, permitted the identification of complexes capable of recognizing and binding a specific ligand (Jensen *et al.*, 1969). Such experiments and



**Figure 14.1 Modular structure of the nuclear receptor family.** Members of the nuclear receptor family contain segments that are conserved to varying degrees among the family members. (A) A schematic structure of a prototypic nuclear receptor is shown. The conserved ligand binding and DNA binding domains are indicated (hatched and filled rectangles, respectively). The relative positions of the activating functions (AF)-1 and -2 within the amino terminus and carboxyl terminus of the receptor proteins are shown. Sequences responsible for the nuclear localization (NLS) of these proteins have been localized to the carboxyl terminal end of the DNA-binding domain. (B) While the relative positions of the individual domains are in most instances maintained, the degree of sequence conservation varies widely. Members of the nuclear receptor family exhibit the highest degree of conservation when the amino acid sequences of the DNA binding domains of the receptor proteins are compared. Lesser degrees of homology are evident between the sequences that comprise the ligand binding domains of the receptors. In the example shown, the predicted amino acid sequences of three different members of the nuclear receptor family are compared. The degree of relatedness is shown for each of the two receptors when aligned with the predicted amino acid sequence of the human glucocorticoid receptor. The amino terminal segments of the receptors differ considerably in size and sequence. The extent of homology is less than 15% when the amino termini of the receptor proteins are compared. (C) In some members of the nuclear receptor family, differences are evident even in the fundamental organization of the receptor proteins. The COUP-TF protein exhibits a structure that is similar to that of the other nuclear receptor protein, with a small amino terminus, a DNA binding domain comprised of two zinc fingers, and a segment corresponding to the ligand binding domains of the steroid receptors (COUP-TF is an orphan receptor). By contrast, although the amino acid sequence of the orphan nuclear receptor, DAX-1, predicts a segment homologous to the ligand-binding domain of other nuclear receptors, the amino terminal segments is comprised of distinctive segments that mediate the binding of this nuclear receptor to DNA (stippled box). SHP, while encoding segments similar to the ligand binding domains of other nuclear receptors, lacks segments that mediate its binding to DNA. (D) Schematic organization of the human glucocorticoid receptor DNA binding domain. The zinc ions serve as nucleation centers for the two “zinc finger” modules of the DNA binding domain. The regions identified by mutagenesis studies as being important for receptor dimerization (D-box) and for target gene specificity (P-box) are indicated. In some members of the nuclear receptor family, carboxyl terminal extensions (CTE) of the DNA binding domain are important determinants of the high affinity binding of the receptor proteins to its DNA targets (the T- and A-boxes).

findings quickly led to similar investigations using a variety of radiolabeled steroid hormones, which resulted in the identification of high affinity receptors capable of specifically binding each of the classic steroid hormones. These studies led several groups to formulate paradigms that have guided hypotheses and experiments in the area of nuclear receptor (NR) structure and function in the ensuing thirty years. According to such models, the receptor proteins are initially synthesized as components of large macromolecular complexes. The ligand binding leads to changes in the composition of these complexes and to the transformation of the receptors to a DNA-binding competent state that facilitates their interaction with target DNA sites within the chromatin (Gorski *et al.*, 1968). The purification of the receptor proteins, coupled with the use of protease digestion and photoaffinity labeling techniques, led to the recognition that distinct portions of the receptor protein were responsible for the binding of ligand and recognition of DNA (Gustafsson *et al.*, 1984, 1987; Rousseau, 1984).

### STRUCTURAL FEATURES OF NUCLEAR RECEPTOR FAMILY MEMBERS

The cloning of the estrogen and glucocorticoid receptors (ER and GR, respectively) permitted the first detailed structure-function analyses to be conducted. The studies performed using cDNAs encoding these receptors (Giguere *et al.*, 1986; Kumar *et al.*, 1987) defined the essential elements of nuclear receptor structure and have proven instrumental in formulating investigations of other members of the nuclear receptor family (summarized by Evans, 1988). Linker scanning and deletion mutagenesis demonstrated that the highly conserved cysteine-rich motif was responsible for mediating the binding of the receptor proteins to DNA (Figure 14.1 A and D). Similar experiments demonstrated that the carboxyl terminal segment of the receptor proteins was responsible for the high affinity binding of ligand (Giguere *et al.*, 1986; Kumar *et al.*, 1987). Additional studies further refined these concepts and defined additional characteristics of the receptor proteins. For example, analysis of mutant receptors in which portions of the DNA binding domains (DBD) of the estrogen and glucocorticoid receptors were exchanged led to the identification of segments within the DBDs that were responsible for dimerization and for discrimination among the nucleotide sequences of target DNA response elements (D- and P-boxes, Figure 14.1D) (Umesono and Evans, 1989; Mader *et al.*, 1989; Danielsen *et al.*, 1989). Additional deletion mutagenesis studies defined regions within the amino terminal and carboxyl terminal segments of the receptor proteins that conferred transcriptional activation properties on heterologous fusion proteins (Figure 14.1A). One activating function (AF-1) was localized to the amino terminus of the receptors and was found to be active when assayed in heterologous systems whether or not ligand was present. By contrast, a second activating function (AF-2) was localized to the hormone binding domain itself and required the binding

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of an agonistic ligand for activity in such systems (Giguere *et al.*, 1986; Kumar *et al.*, 1987; Brou *et al.*, 1993; Parker, 1998). Additional segments within and adjacent to the DBD have been shown to mediate the nuclear localization of the receptor proteins (NLS, Figure 14.1A) (Picard *et al.*, 1990; Ylikomi *et al.*, 1992; Jenster *et al.*, 1993; Zhou *et al.*, 1994; Guiochon-Mantel *et al.*, 1996; Savory *et al.*, 1999).

In addition to permitting detailed studies of the domain structure of the receptors, the cloning of the ER and GR quickly led to the recognition that these receptors contained motifs that were shared among members of a large multigene family. This resulted in an explosion of research that culminated in the cloning of all of the receptors for the classic steroids, as well as those that mediate the actions of thyroid hormone and retinoic acid by 1989 (Evans, 1988). In addition to the identification of the receptors responsible for transducing classic hormonal signals, these experiments had an unanticipated outcome, in that they led to the discovery of a group of proteins that shared structural similarity to members of the steroid-thyroid-retinoic acid receptor family (principally in the segment encoding the DNA-binding domain; Figure 14.1B and C). Although these proteins possess motifs suggesting the capacity to bind ligands, none were readily apparent, and they have been classified in the orphan receptor subgroup of the nuclear receptor family (Evans, 1988; Mangelsdorf *et al.*, 1995; Kliewer *et al.*, 1999; Giguere, 1999). While in most instances these orphan receptors preserved the overall structure of the nuclear receptor family, in some cases proteins have been identified in which only selected domains are present (Figure 14.1C) (Zanaria *et al.*, 1994; Seol *et al.*, 1996; Lalli *et al.*, 2000). For several receptors, ligands that modulate transcriptional activity subsequently have been identified, and these receptors are no longer considered orphans (Heyman *et al.*, 1992; Forman *et al.*, 1998; Kliewer *et al.*, 1998; Lehmann *et al.*, 1998; Blumberg *et al.*, 1998a, b; Giguere, 1999; Janowski *et al.*, 1999; Kliewer *et al.*, 1999; Makishima *et al.*, 1999; Wang *et al.*, 1999; Jones *et al.*, 2000; Moore *et al.*, 2000).

#### DIVERSITY OF THE NUCLEAR RECEPTOR FAMILY: EVOLUTIONARY ASPECTS

As noted above, the DNA binding domain was quickly recognized as a conserved motif in the nuclear receptor family. In addition to the identification of orphan members of the nuclear receptor family in mammals, the conserved nature of this domain has also permitted the identification of large numbers of related genes in other species. This process has been further augmented by the large-scale genome sequencing projects that are ongoing for many organisms.

The comparison of the predicted sequences of such proteins within and among species has permitted a view of how these gene families have evolved (Figure 14.2). One of the most detailed analyses of the evolution of this family are found in the elegant studies of Laudet and colleagues (Escriva *et al.*, 1997; Laudet, 1997). In these experiments, the investigators employed degenerate primers to amplify a highly conserved region of the DNA binding domain from a number of vertebrates and invertebrates. These investigators found that they could amplify and clone segments of DNA from a wide range of metazoans. Of interest, only selected

families of receptors, such as COUP-TF, RXR, and FTZ-F1, could be amplified from some of the more primitive species. These negative experiments could not be explained on the basis of trivial explanations, such as the rapidity of evolution of these organisms. Additional diversification in the number of different orphan receptors appears to be correlated with waves of gene duplication. By contrast, the steroid receptors appear to be relatively recent evolutionary events, without parallels in lower organisms, such as the arthropods. This idea, coupled with the related observation that the capacity to bind ligand is shared among widely divergent members of all six subcategories of nuclear receptor proteins, suggests that the capacity to bind and be regulated by ligand is a relatively recent evolutionary event that arose independently from the evolution and divergence of nuclear receptors.

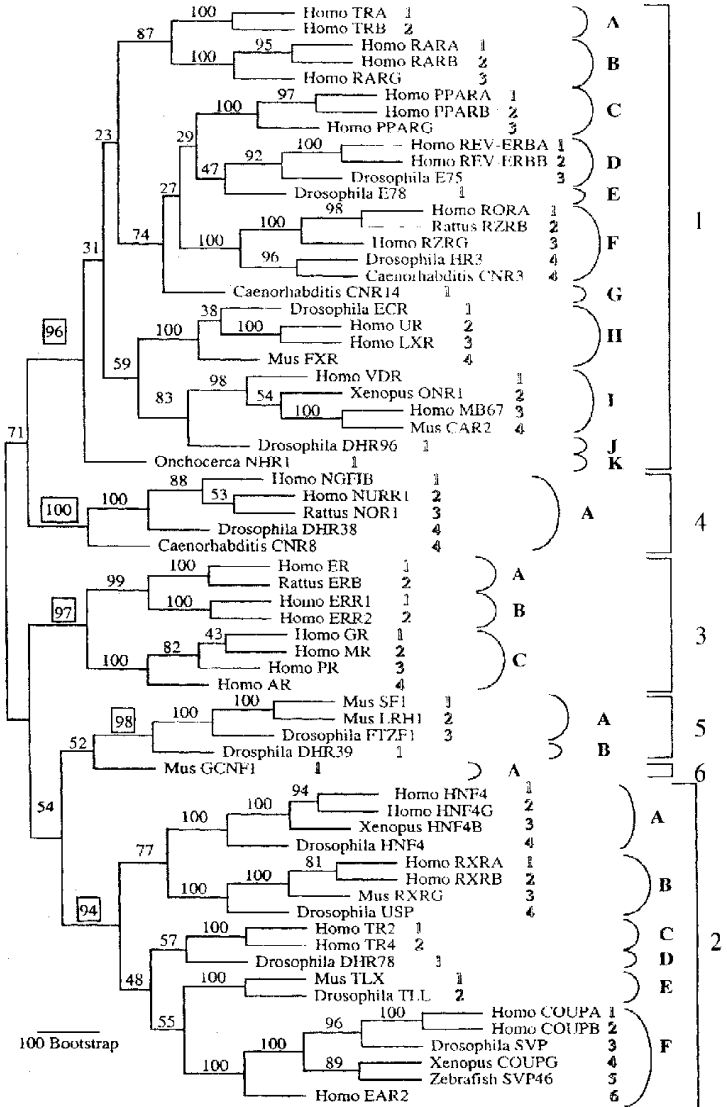
## NOMENCLATURE

As outlined above, the identification and characterization of members of the nuclear receptor family proceeded in a stepwise fashion. Beginning with the isolation and characterization of cDNAs encoding specific steroid receptor family members, the reagents and sequences afforded by these initial clones stimulated the screening to identify cDNAs encoding as yet unknown members of the NR family. For this reason, many of the names and descriptions were derived from characteristics of the cloning process itself or the site of expression. As a result, the nomenclature of the nuclear receptor family has been somewhat chaotic. Recently, several investigators have outlined a more precise nomenclature that is based on phylogenetic relationships between the different members of the nuclear receptor family (Anonymous, 1999). While most investigators still routinely employ the more trivial names, the new nomenclature has permitted a more rational naming process and has been employed increasingly in the nucleotide and protein databases to avoid potential ambiguities.

## MECHANISMS OF NR ACTION

### Chaperone binding and NR synthesis

The characterization of the steroid receptors led to the recognition that their physical characteristics changed dramatically when analyzed in the presence or absence of ligand (Gorski *et al.*, 1968). In the untransformed (unliganded) state, steroid receptors were found to behave as large complexes. Following the binding of ligand, these complexes changed in apparent size and migrated in sucrose density gradients with a much smaller apparent molecular mass (4S, compared to 8–9S in the untransformed state). Purification of the untransformed receptors revealed that proteins capable of binding ligand as well as non-binding proteins were components of the untransformed receptor complex. Biochemical fractionation and purification experiments, particularly those performed to characterize the progesterone (Tuohimaa *et al.*, 1984) and glucocorticoid (Dougherty *et al.*, 1984) receptors, identified a major non-binding component with a molecular mass of approximately 90 kilodaltons. Subsequent characterization using physical (Housley *et al.*, 1985; Catelli *et al.*, 1985) and immunological (Sanchez *et al.*, 1985) methods demonstrated this protein to be



**Figure 14.2 Evolutionary relationships among members of the nuclear receptor family.** An analysis of the predicted protein sequences of members of the nuclear receptor family from mouse, rat, human, nematodes, and *Drosophila* predicts that this diverse group of proteins is derived from a common ancestral protein. The bootstrap analysis of these diverse proteins performed by Laudet *et al.* permits their grouping into six distinct families containing a number of subfamilies and subgroups (Escriva *et al.*, 1997; Laudet, 1997). This type of grouping has permitted an overview of the evolutionary relationships between different members of this large family of transcriptional regulators. This analysis has led Laudet to conclude that the acquisition of ligand binding is a late event in the evolution of this family, as members of distant families exhibit the capacity to bind ligand (e.g., the RXR family members of family 2 B, the steroid receptors of family 3 A and C and the vitamin D receptor of family 1 I). In addition, given the vast diversity of proteins within this family and the trivial nomenclature that has evolved, this systematic family tree has permitted the construction of an unambiguous naming system.

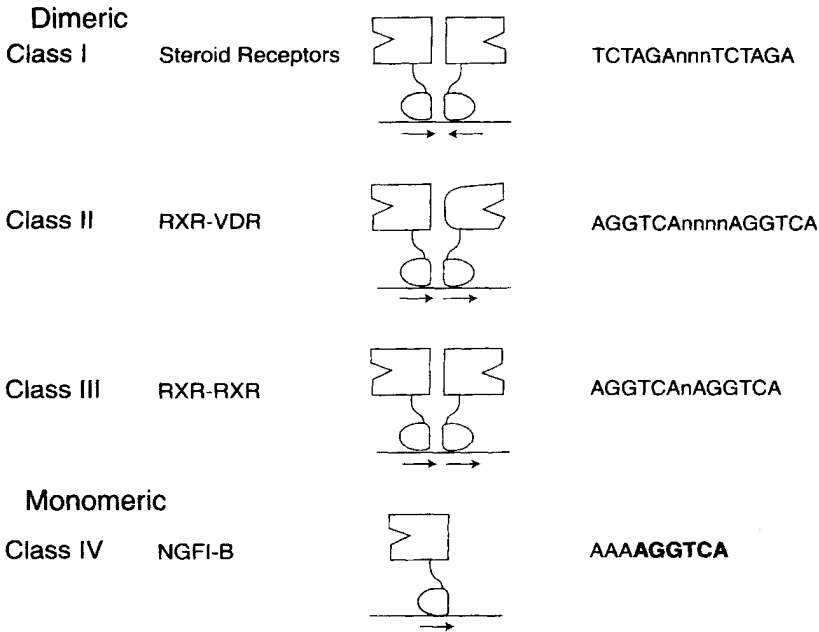
the chaperone, heat shock protein 90 (HSP 90). Further analyses identified this same protein as a component of the untransformed receptor complexes for the androgen, estrogen, and mineralocorticoid receptors (Joab *et al.*, 1984).

While controversy initially surrounded the relevance of HSP 90 to the function of steroid receptors, a variety of investigations have subsequently underscored its critical role in modulating the proper synthesis and activity of members of this family. These studies include *in vivo* experiments demonstrating the functional effects that can be observed when levels of HSP 90 are altered (Picard *et al.*, 1990; Bohen and Yamamoto, 1993), as well as *in vitro* analyses to identify the steps by which the untransformed steroid receptors are assembled into functional complexes containing HSP 90, additional chaperones, and ancillary proteins (Johnson and Toft, 1995; Dittmar *et al.*, 1996; Dittmar and Pratt, 1997). The latter types of studies have characterized the pathways by which these proteins associate with the unliganded steroid receptors. More recent investigations have suggested that chaperones may play roles in receptor recycling and trafficking (Defranco, 2000) and that distinct sub complexes—differing in the complement of ancillary proteins—may exist and subserve specific roles (reviewed in detail in references of Pratt and Toft, 1997).

### DNA binding

Members of the nuclear receptor family possess the capability of binding to specific target DNA sequences to modulate the transcriptional activity of responsive genes. This property is mediated by the conserved DNA-binding segment that is characteristic of the nuclear receptor family. Although the DBD is responsible for the binding of the receptor to its target response elements, the specific response elements that are bound and the fashion in which the target DNA is recognized varies considerably among the different members of the nuclear receptor family (Figures 14.3 and 14.4).

Steroid receptors represent one subgroup within the NR family and were the first for which detailed structural information regarding the interactions between a receptor and its target DNA became available. In general, steroid receptors bind as homodimers to consensus response elements that are inverted palindromes in which a three-nucleotide spacer separates the individual half sites (Figure 14.3, class I). The manner in which the DNA binding domains of steroid receptors interact with target DNA sequences have been studied in detail using NMR spectroscopic and crystallographic methods. The crystal structures of a number of NR-DNA complexes have been solved, including the estrogen and glucocorticoid receptors (Freedman and Luisi, 1993; Schwabe *et al.*, 1993; Gewirth and Sigler, 1995; Kosztin *et al.*, 1997). While differences have been shown to exist in the structures of these different DNA-binding domains, common themes are readily evident. First, as shown for the GR (Figure 14.4A), the two zinc fingers that comprise the DNA binding domain are oriented such that the two elements are nearly perpendicular to one another. In this configuration, the amino terminal zinc finger of each DBD is positioned within the major groove of the target DNA element and makes specific contacts with the DNA-phosphate backbone. Amino acid residues within the P box of the zinc finger (see Figure 14.1D), defined functionally as the residues imparting specificity for target DNA recognition (Umesono *et al.*, 1989; Mader *et al.*, 1989; Danielsen *et al.*, 1989), make direct contacts with specific bases within the target DNA sequence motif. Finally, residues within



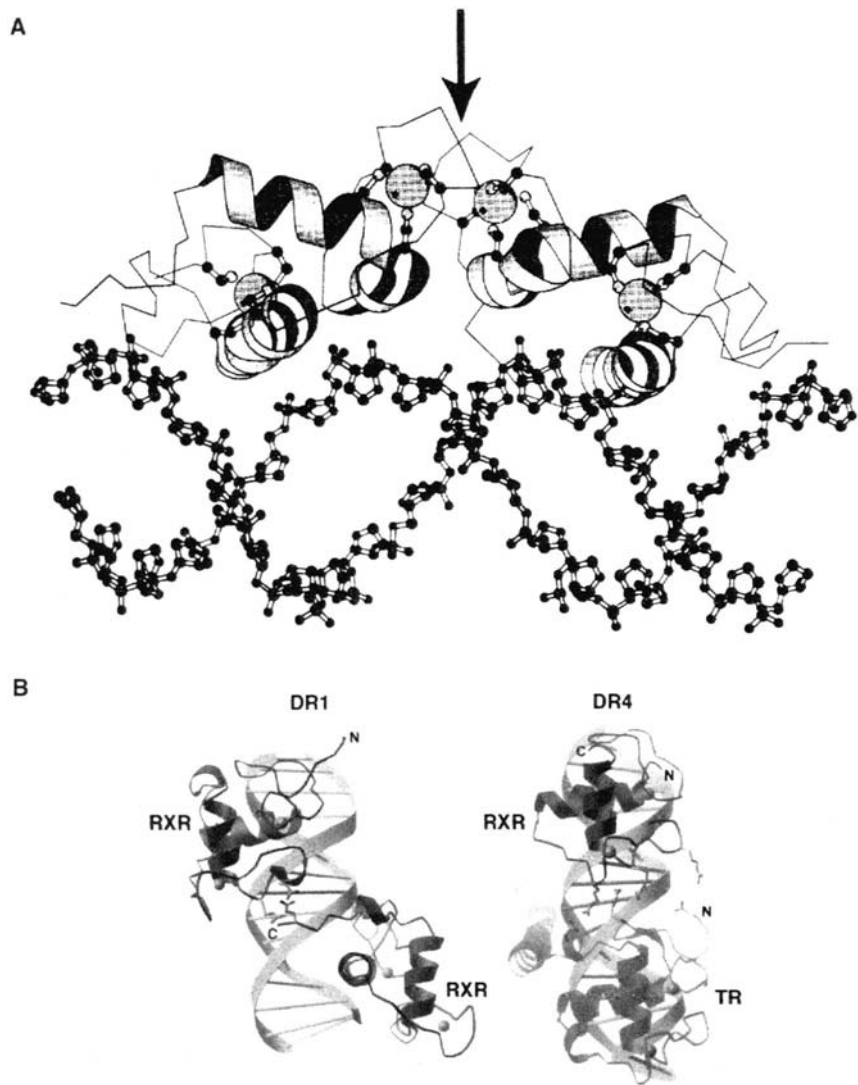
**Figure 14.3 Schematic representations of the different mechanisms by which members of the nuclear receptor family recognize DNA.** In many instances, members of the steroid receptor family (class I) bind to sequences similar to the inverted palindromic sequence shown at right. In this type of recognition sequence, a three-nucleotide "spacer" (nnn) separates the individual half sites. Nuclear receptors with non-steroidal ligands (class II) bind to DNA in association with the obligate heteromerization partner, RXR. Depending the receptor (e.g., vitamin D receptor, thyroid hormone receptor, peroxisome proliferator-activated receptor, or retinoic acid receptor) the spacing between the two individual half sites is distinct. Class III NRs bind as homodimers to their target sequences. Some members of the nuclear receptor family (Class IV) bind to their target DNA sequences as monomers. In these instances, nucleotide residues adjacent to the core response element form a critical component of the recognized sequence. Adapted from Mangelsdorf *et al.*, 1995.

the DBD that had been shown to be functionally important for mediating the dimerization of the receptor on response elements (D box), can be seen as sites of direct contact between the two receptor monomers when bound to DNA in the crystal structures. In aggregate, this combination of protein-protein, protein-DNA interactions, and the exclusion of water molecules appear to comprise the energetic basis for the specific DNA recognition by steroid receptor DNA binding domains (Freedman and Luisi, 1993; Schwabe *et al.*, 1993; Gewirth and Sigler, 1995; Kosztin *et al.*, 1997). It should be noted that while these patterns may be representative of the manner in which many steroid receptors bind to their palindromic response elements, additional patterns of binding have been identified that suggest fundamentally different mechanisms of interaction of the steroid receptors with DNA (Schoenmakers *et al.*, 2000; Verrijdt *et al.*, 2000).

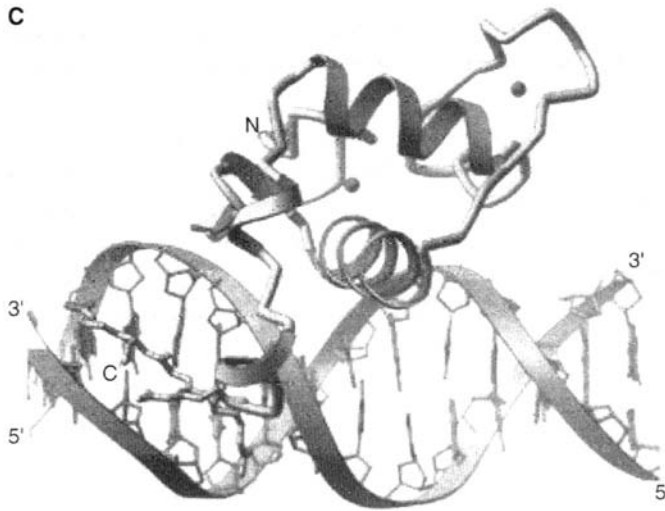
A second subgroup within the NR family is comprised of receptors that require a different binding partner for proper recognition of their respective DNA response elements (Figure 14.3, class II). This class of protein includes a number of diverse members, including the receptors for thyroid hormone (TRs), vitamin D (VDR), retinoids (RARs), and the peroxisome proliferator activated receptors (PPARs). These receptors bind to specific classes of response elements in combination with a common heterodimeric partner, the retinoid X receptor (RXR). Among this group of NRs, specificity as to the DNA elements recognized by the individual receptors are imparted to a considerable extent by the spacing between the individual half-sites, as well as by the nucleotide sequence of the response element themselves (reviewed by Umesono *et al.*, 1991). The individual monomers assemble on the response elements in an asymmetric conformation. In most instances, the RXR DBD occupies the upstream (5') half site, although in selected instances, the RXR DBD can be shown to preferentially occupy the downstream (3') element (Ijpenberg *et al.*, 1997; Rastinejad *et al.*, 2000).

Several generalizations can be made from a consideration of the solved structures available, using the structure of the RXR-TR heterodimer bound to a DR4 element (direct repeats of the half-sites with a 4 nucleotide spacer) as an illustrative example (Figure 14.4B; Rastinejad *et al.*, 1995). First, the overall folding/shape of the core DBD elements, the zinc fingers, is similar between the RXR-TR heterodimer and other nuclear receptor family members, such as the steroid receptors. The spacing between the two half-sites positions the centers of the two DBDs on the same side of the DNA molecule, approximately one full DNA turn apart. As a result, the recognition helices of the two monomers interact with the major groove of the two repeats on the same side of the DNA molecule. While the structures of the core DBDs of both the TR and RXR monomers are similar to that observed in other nuclear receptors, the C terminal extension of the TR projects across the minor groove of the DNA to make both phosphate and base contacts (see Figures 14.1D and 14.4B). The nature of the target DNA imposes an obligate head-to-tail orientation of the RXR and TR DBDs. The spacing and orientation of the two DBDs permit extensive interactions between the two molecules, but owing to the asymmetry of this complex, these extensive interactions are only possible when the RXR molecule occupies the 5' half site. In aggregate, the surfaces by which the two molecules interact with each other and with the DNA response element are dictated by their shapes, by the precise spacing between the two half sites, and by changes in the structure of the DBDs that are induced by their binding to DNA (i.e., compared to structures determined in solution). Although the proteins and response elements differ among various heteromeric complexes (Rastinejad *et al.*, 1995, 2000; Evans, 1988), it is likely that combinations of these same factors will be found to contribute to the specificities of binding that are observed in each of the heterodimers.

The recognition of direct repeat target DNA elements by NR homodimers (Figure 14.3, class III) involves many of the same types of interactions that have been observed in the binding of other nuclear receptors to DNA. The structure of the RXR homodimer assembled on a DR1 motif (direct repeat with a single nucleotide spacer) will serve as the focus of discussion (Figure 14.4B; Zhao *et al.*, 2000). The DBDs are arrayed on the target DNA sequences in a head to tail fashion. The binding of each RXR DNA binding domain to the DR1 element involves the amino terminal recognition helix contacting the bases and



backbone of DNA within the target element. In addition to these features, which are shared with some members of the nuclear receptor family, the carboxyl terminal extension of the DNA binding domain (functionally defined as the T-box) can be seen to assume an extended conformation. Dimerization involves the interaction between residues of this carboxyl terminal extension (of the downstream subunit) with residues of the second zinc finger (from the upstream subunit; see [Figure 14.4](#)). In keeping with the results of biochemical and genetic studies, the half site spacing is found to be critical for the contacts that are observed between the two components of the homodimer.



**Figure 14.4 Recognition of target DNA elements by members of the nuclear receptor family.** (A) The DNA binding domain of the glucocorticoid receptor interacts with its palindromic target DNA element as a homodimer. The amino terminal recognition helix interacts with elements of the sugar phosphate backbone, as well as with specific bases within its recognition sequence. The three nucleotide spacing between the two half sites permits extensive interactions between the two monomers. (B) Some NR DBD hetero- and homodimers recognize direct repeat elements. On the left is the structure of the RXR homodimer bound to the DR1 (direct repeat, 1 nucleotide spacer) element; on the right is the RXR-TR heterodimer bound to the DR4 (direct repeat, 4 nucleotide spacer) response element. Many of the features evident in the binding of the GR DBD to its response element are also found in these structures. Within each dimer, the conformations of the core DBD of each monomer are highly conserved in the organization of the two core alpha helices with respect to the zinc-nucleated modules. While the overall structures considerably differ between homo- and heterodimers, the amino terminal recognition helix centers each dimer on the specific target DNA. The sites and nature of the interactions between the two component monomers are dictated by the spacing of the molecules on their respective target DNAs. In both instances, segments carboxyl terminal to the core DBD of the downstream monomer play an important role in the formation of the dimer interface (see text). (C) As observed in the other NR complexes, the general structure of the core DBD is preserved when monomeric NRs bind to DNA. Specific contacts are made by the amino terminal recognition helix, which are important for binding to specific response elements. In addition to these contacts, however, a carboxyl terminus extension of the NGF I-B DBD (T-box) crosses the DNA backbone permitting more distal elements (the A-box) to interact with the minor groove of DNA. Structure-function studies have established the importance of these latter interactions for the stable binding of such receptor monomers to their target sequences. Reprinted by permission from Meinke and Sigler, 1999.

Several members of the nuclear receptor family have been demonstrated to bind specific response elements as monomers (Figure 14.3A, class IV). While sharing a number of properties in common with other members of the nuclear receptor family, proteins exhibiting this behavior display some differences in the fashion in which they interact with their specific DNA response elements. In several instances, while recognizing a canonical

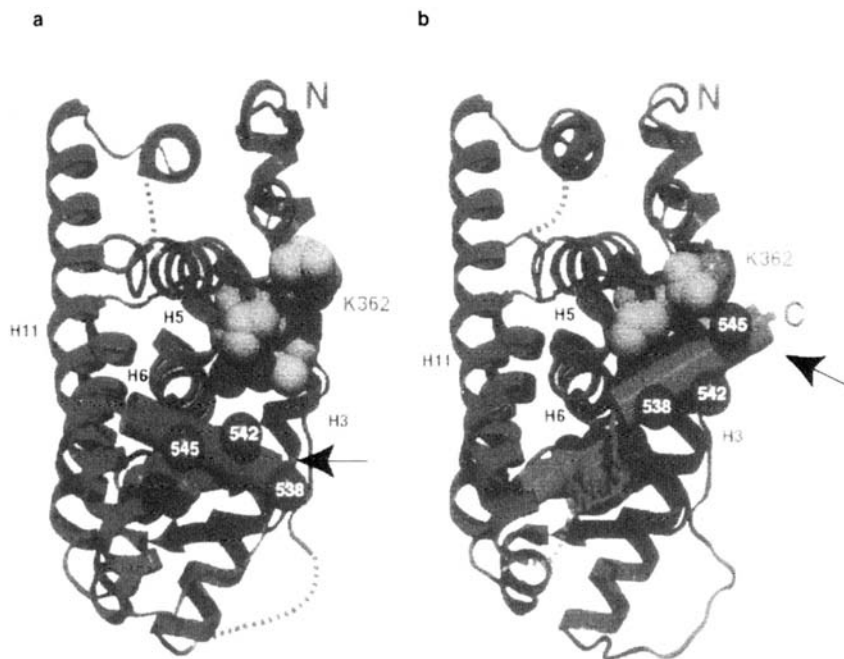


AGGTCA half site, additional nucleotides 5' to the core recognition sequence contribute to the affinity of DNA binding by the nuclear receptor protein. In addition, mutagenesis studies of nuclear receptor proteins have demonstrated that regions carboxyl-terminal to the core DNA binding motif contribute substantially to the avidity of DNA binding. The structures of selected DBDs of this class have now been solved, and that of the orphan receptor NGF I B DNA binding domain illustrates several of these points (Figure 14.4C; Meinke and Sigler, 1999). In the crystal structure, the recognition helix can be seen to make specific contacts within the major groove of the target DNA in a manner analogous to that observed for the interaction of other members of the NR family with DNA. In addition to these interactions, however, the carboxyl-terminal extension (the T- and A-boxes; Figure 14.1D) extend toward the 5' regions of the target DNA, where the A-box elements make direct contact with sequences within the minor groove. Comparisons of the target DNA elements and the primary sequences of members of the NGF I B and SF1 (steroidogenic factor 1) nuclear receptor subfamilies have suggested that similar types of interactions between the carboxyl terminal extension of the DBD and the 5' elements of the target DNA sequence may be characteristic of members of these branches of the nuclear receptor family.

### Ligand binding

Detailed information now exists regarding the structure of the ligand binding domains (LBDs) of diverse members of the NR family (Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995; Brzozowski *et al.*, 1997; Rochel *et al.*, 1997, 2000; Darimont *et al.*, 1998; Nolte *et al.*, 1998; Ribeiro *et al.*, 1998; Shiau *et al.*, 1998; Tanenbaum *et al.*, 1998; Williams and Sigler, 1998; Wurtz *et al.*, 1998; Norman *et al.*, 1999; Pike *et al.*, 1999; Johnson *et al.*, 2000). The reported structures include members of many different subgroups of the NR family. Although the LBD of the TR was the first to be solved (Wagner *et al.*, 1995), owing to the focus of this volume, the structure of the ER alpha LBD is discussed as representative of such structures (Figure 14.5).

The LBD of the estrogen receptor was the first of the steroid receptors to be solved (Brzozowski *et al.*, 1997). It was found to be composed of 12 alpha helices arranged in a "layered helical sandwich", as had been observed in the structures of other NR LBDs that had been solved (Figure 14.5; Bourguet *et al.*, 1995; Renaud *et al.*, 1995; Wagner *et al.*, 1995). In this "sandwich", a central core is formed by three helices (helices 5/6, 9, and 10). This central layer is located between two additional layers made up of helices 1–4 and helices 7, 8, and 11. The position of helix 12 will be described below. The estrogen-binding pocket occupies a relatively large part of the hydrophobic core of the structure and is sequestered from the external environment. Specific ligand recognition is achieved through a combination of specific hydrogen bonds (involving the phenolic hydroxyl group and the 17 $\beta$ -hydroxyl group of the ligand), as well as a number of hydrophobic contacts (between the steroid nucleus and hydrophobic residues that line the ligand-binding pocket of the receptor). The overall size of the ligand-binding cavity (approximately 450 cubic angstroms) is approximately twice the molecular volume that is occupied by the estradiol molecule itself. In the estradiol-liganded ER ligand binding domain, helix 12 is positioned in a fashion



**Figure 14.5 Structures of nuclear receptor LBDs.** Structures of the ligand-binding domain of the human estrogen receptor- $\alpha$  complexed to estradiol (a) or to Raloxifene (b) have been solved. In the agonist-bound complex, helix 12 is tightly opposed to the helices that comprise the ligand-binding pocket. As a result, the surfaces responsible for the recruitment of nuclear receptor coactivators, such as SRC-1, are freely accessible. By contrast, the binding of Raloxifene within the ligand-binding pockets prevents helix 12 from assuming the same configuration. Instead, helix 12 interacts with the same surfaces that are available for interacting with nuclear receptor coactivators in the agonist-bound complex. The arrows in the two panels indicate the positions of the terminal helix 12 in the two crystal structures. Reprinted by permission from *Nature* **389**, 753–758, copyright 1997 Macmillan Magazines Ltd.

that causes it to be closely opposed to the framework of the LBD “sandwich” and is positioned adjacent to helices 3, 5/6, and 11. As noted by the authors, although this helix could be seen to make no direct contacts with ligand, helix 12 was positioned in a fashion analogous to a “lid” that serves to “seal” the ligand-binding pocket. These authors noted that this position of helix 12 was similar to that observed in the other ligand-bound nuclear receptors and postulated that this structure was necessary for the generation of an active AF-2 domain capable of recruiting NR coactivators.

In this same report (Brzozowski *et al.*, 1997), these authors reported the structure of the ER complexed to an antagonist (Raloxifene), and the contrasts evident between the structures of the agonist-bound and antagonist-bound LBDs were provocative. Comparison of these two structures clearly implicated conformational changes of the terminal helix of the LBD in mediating the different biological activities of these two complexes. In contrast

to the position of helix 12 over the ligand binding pocket that is observed in the estradiol-bound structure, the binding of Raloxifene within the ligand binding pocket prevents helix 12 from sealing the ligand binding cavity and instead forces a rotation of helix 12 by approximately 130 degrees (Figure 14.5). Subsequent analyses of agonist- and antagonist-bound estrogen receptor complexes have reinforced the basic concepts as to the importance of the position of the helix 12 in response to agonists and the role of this conformation in the recruitment of coactivators by the AF-2 surface (Shiau *et al.*, 1998). When complexed to an agonist, the position of helix 12 has been shown to be critical to the formation of surfaces that are required for the recruitment and binding of the P160 coactivators. In the antagonist-bound LBD, by contrast, helix 12 occludes these surfaces by interacting with the hydrophobic groove that serves as the pocket to which the NR coactivators bind (Shiau *et al.*, 1998) (see below).

While differing in the size of ligand-binding pockets and the specific contacts that lead to the different ligand specificities, structures of other ligand-bound nuclear receptors reinforce the concepts that have emerged in the study of agonist- and antagonist-bound ER- $\alpha$  (Nolte *et al.*, 1998; Ribeiro *et al.*, 1998; Tanenbaum *et al.*, 1998; Williams and Sigler, 1998; Rochel *et al.*, 2000).

### Coactivators and corepressors

In early experiments, it was noted that the levels of reporter gene activation by nuclear receptors varied dramatically as a function of the conditions under which the transient transfections are performed (i.e., “squenching”). In these experiments, the transcriptional activity of an activated nuclear receptor was found to be diminished by the presence of a second activated nuclear receptor, even though the second receptor did not bind to the promoter being studied. This receptor interference led to the idea of limiting, common factor(s) that mediate the interaction of nuclear receptors with the general transcription machinery (Tasset *et al.*, 1990; Shemshedini *et al.*, 1992; Hoeck *et al.*, 1992; Webb *et al.*, 1992). That this “squenching” effect characterized the behavior of many different nuclear receptors gave the first indications of the presence of coregulatory factors and their potential roles in modulating nuclear receptor function.

The factors are now known as nuclear receptor coactivators and corepressors. By definition, these coregulators interact with nuclear receptors and alter transcription of target genes. In general, coactivators are recruited by agonist-bound receptors and corepressors interact with unliganded or antagonist-bound receptors. Even though most have no intrinsic transcriptional activity by themselves, some coregulators have been demonstrated to interact with components of the general transcription machinery, either as individual polypeptides or as components of a stable complex. Finally, many NR coregulators have been shown to interact with mediators of other cell signaling pathways or protein degradation pathways. Although an increasing number of distinct coactivators and corepressors are being identified and their physiologic roles are being characterized, only a brief overview of a major class of coregulators is presented here (see Collingwood *et al.*, 1999; McKenna *et al.*, 1999; Xu *et al.*, 1999; Leo and Chen, 2000; Robyr *et al.*, 2000 for detailed reviews).

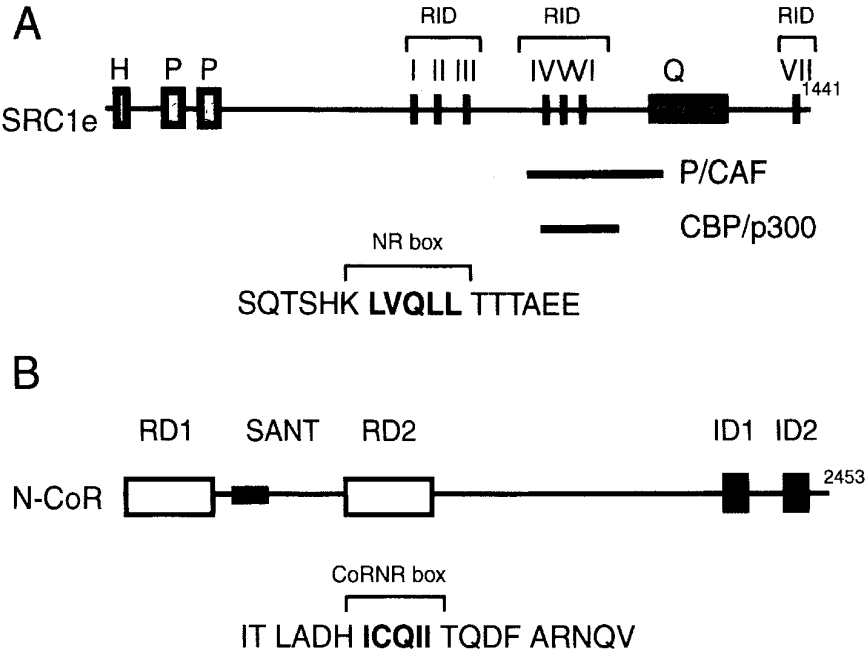
### Coactivators

Initially, studies to define the intermediary proteins that participate in the direct actions of activated nuclear receptors focused on the use of assays, such as two-hybrid screening, to identify proteins that interacted with the ligand binding domains of several steroid receptors. Among the first proteins isolated using such methodologies was steroid receptor coactivator-1 (SRC-1; Onate *et al.*, 1995). This protein was identified as interacting with the LBD of the human progesterone receptor in an agonist-dependent manner. Transfections of cDNAs encoding SRC-1 demonstrated its capacity to augment the activity of the agonist-bound progesterone receptor. Parallel studies conducted to identify factors important for the function of the activated glucocorticoid and estrogen receptors identified other proteins that were related structurally to SRC-1. These are now considered to comprise a gene family termed the SRC family or P160 (proteins of 160kD) family of nuclear receptor coactivators (reviewed in detail by Leo and Chen, 2000). These proteins have been shown to serve roles beyond the modulation of NR function, as some P160s have been shown to effect the activity of other transcription factors, including AP-1, NF- $\kappa$ B and serum response factor (Kim *et al.*, 1998; Lee *et al.*, 1998; Na *et al.*, 1998).

The P160 family of steroid receptor coactivators consists of proteins encoded by at least three distinct genes (SRC-1, SRC-2/TIF2/GRIP2, and SRC-3/ACTR/ AIB-1/pCIP/ RAC1). Each gene has been shown to give rise to multiple differentially spliced mRNAs that encode proteins with numerous functional and structural similarities. For example, each was shown to specifically associate with the agonist-bound form of their respective receptors (each SRC family member can interact with multiple nuclear receptors). As their name implies, steroid receptor coactivators augment the activity of the agonist-bound receptor in functional assays. In addition, overexpression of P160 proteins is capable of eliminating nuclear receptor squelching in transient transfection assays.

In terms of structural or motif similarities, P160 proteins are characterized by the presence of multiple LXXLL motifs (L represents leucine and X represents any amino acid; also called NR boxes (nuclear receptor boxes); Figure 14.6A). Different NR boxes are important for determining the specificity of nuclear receptor-coactivator interactions. As mentioned above, crystallographic studies have begun to show that the coactivator binding site in the nuclear receptor LBD is exposed in the presence of agonist and is capable of interacting with LXXLL motifs. In contrast, the coactivator binding site in the nuclear receptor is physically blocked (by helix 12) in antagonist-bound or unliganded nuclear receptors. Although many of the SRC family members were originally identified by their interaction with nuclear receptor AF-2/LBD domains, some also interact with AF-1 in the amino terminal regions of some nuclear receptors (Ma *et al.*, 1999; Alen *et al.*, 1999).

The presence of a basic helix-loop-helix domain and multiple Per/Arnt/ Sim (PAS) domains at the amino terminal portion of P160s suggests the possibility of additional cross-talk with other proteins containing PAS domains or other nuclear receptors (Figure 14.6A). The functional significance of this remains to be determined. SRC proteins already have been shown to interact with components of the general transcription machinery (such as the TATA binding factor (TBP) and transcription factor IIB (TFIIB)) and with other coregulatory factors (such as p300/CREB binding protein (CBP) and p300/CBP associated



**Figure 14.6 Structures of representative coactivators and corepressors.** As outlined in the text, a number of different proteins capable of acting as NR coactivators and corepressors have been described. The structures of SRC-1 and NCoR are depicted here as representative examples. (A) SRC-1e is one of several isoforms derived from the SRC-1 gene by alternative splicing. It is representative of a gene family, the P160 coactivators, which also includes GRIP-1 and ACTR. The predicted amino acid sequence of SRC-1e includes seven LXXLL motifs that mediate the interaction of SRC-1 with the agonist bound NRs (I-VII). In addition, a glutamine-rich segment is predicted (Q). The segments that have been shown to mediate the recruitment of P300/CBP and P/CAF are indicated. As depicted, SRC-1 also contains motifs in common with the basic helix-loop-helix (H) and PAS (P) domain families of transcription factors. The functions of these latter elements remain to be defined. (B) NCoR is a large protein that shares significant sequence homology with SMRT (overall sequence identity 34%). Two segments localized to the carboxyl terminus of the protein mediate its interaction with the NR (interaction domains, ID). Two regions at the amino terminus mediate the repressive effects on gene transcription (repression domains, RD). The SANT (SWI3, ADA2, NCoR, and TFIIIB) domain is a putative DNA binding domain that is found in some proteins involved in transcriptional regulation or chromatin remodeling.

factor (P/CAF); see below) (Takeshita *et al.*, 1996; Lazennec *et al.*, 1997; Ikeda *et al.*, 1999).

In addition to structural similarities, P160 family members are believed to possess similar functional roles. Each is believed to participate in the modulation of gene activity in at least two ways. The first is by effecting an alteration of the structure of the chromatin surrounding the sites of transcription initiation. At least a portion of this activity is believed to be due to the histone acetyltransferase (HAT) activities intrinsic to the P160 coactivators themselves, or contributed by proteins recruited by the coactivators (e.g., CBP, p300 and P/

CAF). Acetylated histones are associated with regions of transcriptionally active DNA (Wolffe and Pruss, 1996). In addition, the P160s are believed to modulate the stability of the preinitiation complex itself, leading to enhanced rates of transcription initiation (Jenster *et al.*, 1997).

Two types of experiments have suggested that at least partial functional redundancy exists between different members of the SRC family (mainly SRC-1 and SRC-2). First is the analysis of mice with a targeted deletion of SRC-1. These animals are viable, fertile, and only exhibit partial steroid and thyroid hormone resistance (Xu *et al.*, 1998; Weiss *et al.*, 1999). At the same time, SRC-2 mRNA levels are elevated and SRC-3 mRNA levels do not change in mice that lack SRC-1 when compared to mRNA expression levels detected in mice with intact SRC-1 genes (Xu *et al.*, 1998). This potentially means that elevated expression of SRC-2 may serve to partially compensate for the absence of SRC-1. Secondly, although microinjection of antibodies specific for SRC-1 (but not SRC-2) was able to disrupt retinoic acid receptor-dependent activation of a reporter gene, co-microinjection of expression plasmids encoding SRC-1 or SRC-2 (but not SRC-3) was able to rescue transcription of the reporter gene in cells immunodepleted of SRC-1 (Torchia *et al.*, 1997). Despite such observations, other studies of mice with disrupted SRC-3 genes have suggested that while the functions of P160s may be overlapping in some instances, in others they may serve distinct roles (Xu *et al.*, 2000).

The preceding discussion has been focused on the P160 family of coactivators, owing to the breadth of studies that have been conducted. In addition to these studies, however, a number of other proteins have been identified as exhibiting behaviors characteristic of the nuclear receptor coactivator in functional assays (Baudino *et al.*, 1998; Moilanen *et al.*, 1998; Na *et al.*, 1998; Yeh *et al.*, 1998; Alen *et al.*, 1999; Brady *et al.*, 1999; Fujimoto *et al.*, 1999; Hsiao and Chang, 1999; Kang *et al.*, 1999; Lee *et al.*, 1999; Monden *et al.*, 1999; Monteno *et al.*, 1999; Kirn *et al.*, 1999; Li *et al.*, 1999; Endoh *et al.*, 1999; Caira *et al.*, 2000; Huang and Stallcup, 2000; Knutti *et al.*, 2000; Muller *et al.*, 2000; Poukka *et al.*, 2000; Tcherepanova *et al.*, 2000; Vega *et al.*, 2000; Zhu *et al.*, 2000). More extensive reviews on the general topic of coactivators can be found in a number of recent review articles (McKenna *et al.*, 1999; Xu *et al.*, 1999; Robyr *et al.*, 2000).

### Cointegrators

As described above, nuclear receptors can recruit SRC family members in response to agonists. Both receptors and coactivators can also interact with p300/CBP or P/CAF, which represent a class of NR coregulator often referred to as cointegrators (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996; Chen *et al.*, 1997; Fonsdal *et al.*, 1998). The term "cointegrator" refers to the idea that they are transcriptional coregulatory proteins and that they are involved in regulating, and potentially integrating, transcription from numerous signaling pathways. They were originally identified as important for transcription by their interaction with the adenoviral E1A protein (p300), CREB (CBP), or p300/CBP (P/CAF) (Chrivia *et al.*, 1993; Eckner *et al.*, 1994; Kwok *et al.*, 1994; Yang *et al.*, 1996), but they also interact with many other classes of transcription factors (e.g., p53, NF- $\kappa$ B,  $\beta$ -catenin, GATA proteins, SMAD proteins and ETS transcription factors) and components of the general transcription

machinery (e.g., TAFs) (Abraham *et al.*, 1993; Avantaggiati *et al.*, 1997; Perkins *et al.*, 1997; Boyes *et al.*, 1998; Hecht *et al.*, 2000; Kirn *et al.*, 2000; Papoutsopoulou *et al.*, 2000; Wada *et al.*, 2000). Having intrinsic histone acetyltransferase activity themselves, CBP, p300 and P/CAF may be altering local chromatin structure (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996), but additional changes may result from the recruitment of other proteins involved in chromatin remodeling (e.g., RNA helicase A; Nakajima *et al.*, 1997). The broad spectrum of interactions suggests possible cross-talk, interference, or amplification of cellular signals, which will be effected by cell-specific expression and expression levels of both coregulatory proteins and transcription factors.

### *Corepressors*

The discovery of proteins capable of acting as corepressors of nuclear receptor function stemmed from observations made in the process of characterizing the activities of cDNAs encoding the thyroid hormone and retinoic acid receptors. In cotransfection experiments, it was observed that the introduction of a cDNA encoding the TR or retinoic acid receptor (RAR) led to a decreased basal activity of model responsive genes (Sap *et al.*, 1986; Weinberger *et al.*, 1986). The addition of ligand relieved this inhibition and led to a further stimulation of gene activity above that observed in the absence of transfected TR or RAR cDNAs. Subsequent investigations traced the sequences responsible for these effects to specific regions within the TR and RAR ligand-binding domains, and demonstrated that transfer of these segments to heterologous fusion proteins conferred similar inhibitory properties on the resulting fusion proteins.

Biochemical and genetic approaches ultimately identified two related proteins (nuclear receptor corepressor (NCoR) and silencing mediator of the retinoid and thyroid hormone receptors (SMRT)) that interacted with these segments of the thyroid and retinoid receptors and which were responsible for mediating the inhibition of target genes in the absence of ligand (Figure 14.6B; Chen *et al.*, 1995; Horlein *et al.*, 1995; Ordentlich *et al.*, 1999). The sequences of NCoR and SMRT cDNAs from humans predict large proteins of 2440 and 2507 amino acids in length, respectively. Comparison of the two amino acid sequences predicts proteins with similar organizations and motifs. While sequence alignment reveals similarity throughout the entire lengths of the proteins, the regions of highest homology are localized to the amino terminal regions and correspond to segments implicated (in NCoR) in the binding of Sin3A, which is thought to be an intermediary protein linking nuclear receptors to histone deacetylases (HDACs; hypoacetylated histones are associated with transcriptionally inactive DNA). Additional highly conserved segments are highly homologous to the SANT (SWI3, ADA2, NCoR, TFIIB) domains identified in several proteins involved in transcriptional regulation or chromatin remodeling (Aasland *et al.*, 1996). Functional analyses have revealed that each protein is modular with specific regions responsible for mediating the interaction with nuclear receptors (CoRNR boxes; Figure 14.6B; Hu and Lazar, 1999; Nagy *et al.*, 1999) and for the assembly of protein complexes that mediate repression (repression domains (RDs); see below). A number of variants derived from alternative splicing have also been described (Ordentlich *et al.*, 1999).

Initial insights into the fashion by which these corepressor proteins mediate the repression of target genes explored mechanisms established in the analysis of gene repression exerted by the Mad proteins (summarized by Pazin and Kadonaga, 1997). These studies demonstrated that the corepressors participated in the recruitment of multiprotein complexes containing mSin3 and HDAC1 and that this repression is mediated via the specific recruitment of histone deacetylase activities (HDACs) (Nagy *et al.*, 1997; Heinzel *et al.*, 1997; Alland *et al.*, 1997). Subsequent experiments have shown the existence of at least seven different HDACs that fall into two different families (based on their amino acid sequence similarities). In addition to such mSin3 containing complexes, recent experiments have demonstrated that members of one class of HDACs interact with specific domains in NCoR and SMRT to modulate repression by the formation of distinct repressive complexes (Huang *et al.*, 2000; Kao *et al.*, 2000). Additional studies have established that NCoR and SMRT are important to the inhibitory effects exerted by ligands capable of acting as steroid receptor antagonists as well (Smith *et al.*, 1997; Jackson *et al.*, 1997) and that the specific levels of corepressor expressed in cells can modulate the levels of repression that are observed (Soderstrom *et al.*, 1997).

In addition to the roles played by NCoR and SMRT in the repression of gene expression, additional proteins have been described that serve to antagonize activation by members of the nuclear receptor family (Burns *et al.*, 1994; Dedhar *et al.*, 1994; Wheeler *et al.*, 1995; Zamir *et al.*, 1997; Haataja *et al.*, 1998; Montano *et al.*, 1999; Altincicek *et al.*, 2000). In general, the pathways by which these molecules antagonize receptor function are either undefined or are distinct from those implicated in the function of NCoR and SMRT. Additional information can be found in a number of excellent reviews on this topic (Hu and Lazar, 2000; Xu *et al.*, 2000).

## CHROMATIN

The link between alterations of chromatin structure and gene expression has long been recognized. Studies examining the activation of a model steroid-responsive gene (mouse mammary tumor virus (MMTV) promoter) demonstrated that activation of this responsive element was associated with changes in the nucleosomal structure surrounding the site of transcriptional initiation (Cordingley *et al.*, 1987; Truss *et al.*, 1995). As the diverse natures of the NR coregulators have become increasingly apparent, the importance of their role in chromatin remodeling has become increasingly evident. In addition to the enzymatic activities associated with coactivator and corepressor complexes (e.g., HAT and HDAC activities) (Pazin and Kadonaga, 1997; Kozus *et al.*, 1998), additional multiprotein complexes are thought to be involved in altering the chromatin structure of promoters of genes responsive to nuclear receptors (Ichinose *et al.*, 1997; Ostlund Farrants *et al.*, 1997; Bourachot *et al.*, 1999; Wade and Wolffe, 1999; Wallberg *et al.*, 2000).

The SWI/SNF complex from yeast and the homologous BRG1/BAF complex from mammals represent one such complex involved in chromatin remodeling (reviewed by Sudarsanam and Winston, 2000; Wade and Wolffe, 1999). This family of proteins consists of 12 (yeast) to 21 (nematodes) members. Some components of these macromolecular structures have similarities to ATPases, actin, and other cytoskeletal proteins, but the



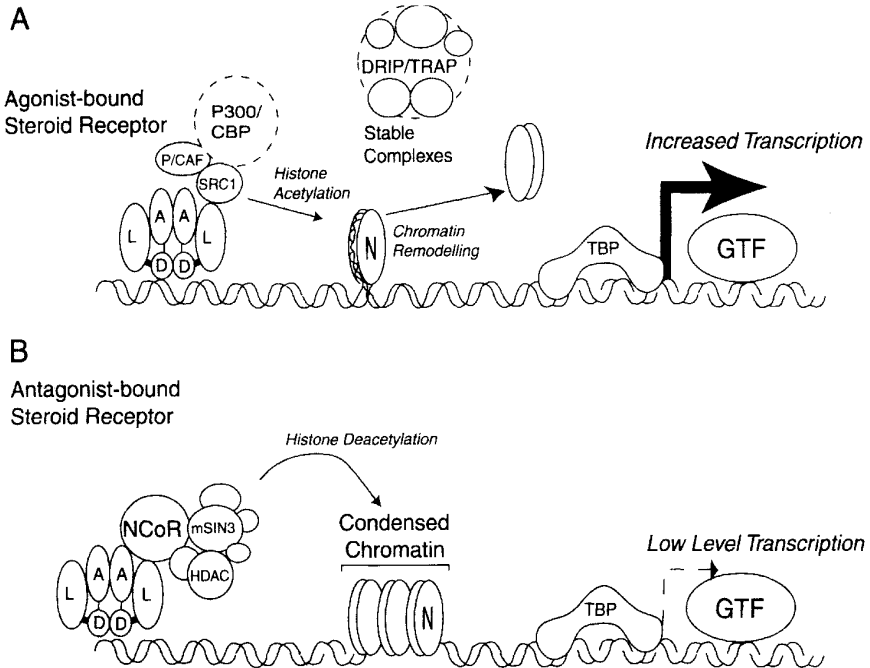
mechanism of action is still not well characterized. Nevertheless, BRG1/BAF complexes are recruited as coactivators by ligand-bound GR, ER, and RAR (Chiba *et al.*, 1994; Ichinose *et al.*, 1997; Ostlund *et al.*, 1997). In contrast, this multisubunit complex also is linked to phospholipid signaling pathways and is involved in transcriptional repression of other classes of transcription factors by association with histone deacetylase activity.

### STABLE COMPLEXES

Perhaps one of the most intriguing developments that has taken place in the last two years in the NR field has been the recognition that distinct classes of proteins interact with different members of the nuclear receptor family and their complexes at different points in the process of transcription. While much information has emerged from the study of the P160 family of coactivators, the characterization of the protein complexes that interact with the ligand binding domains of the activated thyroid hormone and vitamin D receptors has led to the emergence of a different view of the complexes that assemble in response to the binding of ligand. Studies from the Roeder laboratory demonstrated that when the ligand binding domain of the thyroid hormone receptor is expressed in cells and then purified using epitope affinity chromatography, it is found to be associated with a large macromolecular complex composed of multiple polypeptides (TRAPs: thyroid receptor associated proteins) (Ito *et al.*, 1999). Independently, Freedman and co-workers were able to demonstrate that a complex containing a similar complement of polypeptides assembled on the ligand-binding domain of the vitamin D receptor in a ligand-dependent manner (DRIPs: vitamin D receptor interacting proteins) (Rachez *et al.*, 1999). Although similar, each of these complexes is composed of approximately 15 distinct polypeptides. Despite the large and complex nature of these macromolecular complexes, in each instance the complex appears to be tethered to the ligand binding domains via LXXLL motifs contained within a single subunit: DRIP205/TRAP220.

Somewhat unexpectedly, the identification of the individual polypeptides had additional implications for gene activation by these complexes. Several of the DRIP/TRAP subunits are homologous to other macromolecular complexes that have been associated with the activation of genes (Ito *et al.*, 1999; Rachez *et al.*, 2000). Several are analogous to proteins that are components of the Mediator complex, which is known to associate with RNA polymerase II. Furthermore, it is clear that other classes of activators (e.g., Sp1, NF- $\kappa$ B, SREBP-1a and VP16) also recruit complexes containing components of the DRIP/TRAP complexes. Finally, it appears that several DRIP/TRAP subunits can be shown to associate with other SRB-associated complexes, such as NAT and SMCC. As a whole, such findings suggest a certain commonality to the activation of transcription of a wide range of genes (Chiba *et al.*, 2000; Rachez and Freedman, 2000; Ito *et al.*, 2000).

Despite this emerging commonality, a number of important issues defy explanation. Probably the most problematic issue raised by the studies conducted to this point is the absence of proteins possessing acetyl transferase activities in the DRIP/TRAP complex. As this enzymatic activity process has been associated with the process of chromatin remodeling, the absence poses some conceptual hurdles to linking the functions of these complexes to the processes that have been inferred by the activities of the P160



**Figure 14.7 Coactivators and corepressors in the action of nuclear receptors.** A general model for the activities of coactivators and corepressors in the modulation of the transcription of responsive genes by nuclear receptors is depicted using steroid receptors as representative members of this family. (A) The binding of an agonist bound steroid receptor to specific sequences adjacent to the site of transcription initiation of a responsive gene recruits coactivator complexes containing proteins such as SRC-1, p300/CBP and DRIPs/TRAPs (the order or competition among these different proteins for NR binding is unclear at this time). Enzymatic activities contained within these complexes (e.g., histone acetyl transferase) modify the local chromatin structure. In some instances, these modifications may result in large-scale alterations of chromatin organization. These changes make the transcription unit more accessible to the assembly and stability of transcription initiation complexes and results in an increase in the rate of transcription. (B) The binding of an antagonist to nuclear receptors results in the recruitment of protein complexes containing corepressors such as NCoR and SMRT. The enzymatic activities (e.g., deacetylases) associated with these corepressor complexes, which include SINS and HDACs, leads to a condensation of chromatin structure and a decreased level of gene transcription (A: amino terminus of NR with activation functions; D: DNA binding domain of NR; L: ligand binding domain of NR; N: nucleosome; TBP: TATA binding protein; GTF: general transcription factors).

coactivators. While it is not clear exactly how these two classes of proteins (P160s and DRIP/TRAP complexes) relate to one another in the process of gene activation by members of the nuclear receptor family, most current models propose a sequential, step wise participation by such complexes (Figure 14.7A). This view would suggest that the binding of a ligand by the receptor proteins first recruits complexes containing a member of the

P160 family of coactivators, or another protein capable of recruiting HAT activity. This enzymatic activity acts to modify chromatin structure at the site of complex recruitment and results in an "opening" of the compacted chromatin structure. It is possible that subsequent to these initial steps, distinct complexes such as the DRIP/TRAP complexes are recruited via the DRIP205/TRAP220. These changes may result for the destabilization of the initial complexes that are formed, perhaps by cofactor acetylation, such has been described for the coactivator ACTR (SRC-3/AIB-1/pCIP/RAC1)(Chen, 1999). A critical focus of future experiments will be to determine whether both classes of complexes are assembled sequentially on responsive promoters or whether distinct classes of genes are responsive preferentially to the different types of complexes.

#### DIVERSITY OF MECHANISMS IMPLICATED IN STUDIES OF COREGULATOR FUNCTION

The majority of the information summarized above can be placed into a conceptual framework in which nuclear receptors recruit specific coactivators or corepressors to specific DNA sequences within or adjacent to regulated genes. This recruitment initiates a cascade of events that regulate the global structure of chromatin and the rate of transcription initiation (Figure 14.7).

By contrast, a number of molecules have been implicated in the regulation of nuclear receptor function that do not easily fit yet into such an overall regulatory scheme. These include enzymes (which are involved in methylation or degradation of proteins) and RNA molecules (whose precise role has yet to be defined). In most instances, these effectors have been implicated by their identification by the use of two-hybrid screens. Using this methodology, workers in the Stallcup laboratory identified a protein methyltransferase which interacted with the human glucocorticoid receptor ligand-binding domain (Chen *et al.*, 1999). Examination using a combination of *in vitro* and functional assays suggested that this protein was capable of modulating functional activity of the human glucocorticoid receptor. To this point, this observation is restricted to the glucocorticoid receptor.

An equally unlikely candidate was identified in two-hybrid assays using the progesterone receptor ligand-binding domain. These experiments identified a component of the ubiquitin ligase as a nuclear receptor coactivator (Nawaz *et al.*, 1999). Subsequent experiments demonstrated that this coactivation function was independent of ubiquitin ligase activity of the identified protein, suggesting that this protein ligase serves dual roles. In contrast to the observations related to the behavior of protein methyltransferases as coactivators, studies of other receptors have suggested that ubiquitin ligases may play a role in the modulation of the function of several nuclear receptors. In some instances, the regulation of NR activity appears to be mediated by effects similar to nuclear receptor coactivators (Poukka *et al.*, 1999; Sternsdorf *et al.*, 1999). In others, the attenuation of nuclear receptor function results from the destruction of the receptor itself (Syvala *et al.*, 1998; Li *et al.*, 1999; Nawaz *et al.*, 1999; Boudjelal *et al.*, 2000; Lange *et al.*, 2000; Ma and Baldwin, 2000).

Using a two-hybrid-screening assay O'Malley and co-workers identified a partial cDNA, SR-A, which exerted effects as a coactivator on a number of different steroid receptors (Lanz *et al.*, 1999). Although the cDNA was identified in a typical two-hybrid screen,

exhaustive analysis by these authors suggested that the isolated cDNA functioned as an untranslated RNA (careful experimentation failed to identify a functional open reading frame). Despite the marked differences in its character, functional experiments using transfection assays demonstrated that SR-A was capable as acting as a coactivator for a number of different steroid receptors. Furthermore, using gradient centrifugation techniques this RNA was shown to be in high molecular weight complexes containing other prototypic coactivators. Although some additional studies have been conducted which have implicated SR-A expression in the biological behavior of selected breast cancers (Leygue *et al.*, 1999), the mechanisms by which this RNA exerts its effects as a coactivator and its physiologic role remain to be defined.

### ALTERNATE MECHANISMS OF GENE REGULATION

Much of the preceding discussion has been focused on the regulation of transcription by the binding of a nuclear receptor to specific sequences within a gene. As a result of this binding, coregulatory proteins are recruited which serve to alter the state of the chromatin surrounding the site of transcription initiation and influence the assembly of stable transcriptional initiation complexes (Figure 14.7). While a great deal of attention has been paid to unraveling the steps by which nuclear receptors directly modulate the activity of responsive genes via such mechanisms, additional pathways of gene regulation by NRs also have been identified.

Among the first examples of such regulation stemmed from investigations of the inhibition of gene activity by glucocorticoids (Diamond *et al.*, 1990; Schule *et al.*, 1990; Yang-Yen *et al.*, 1990; Lucibello *et al.*, 1990; Shemshedini *et al.*, 1991; Kerppola *et al.*, 1993). A number of laboratories noted that the liganded glucocorticoid receptor was capable of negatively regulating a number of native or artificial genes. While diverse, these genes shared a common attribute in that in each instance an AP-1 site was identified as an important functional feature of the promoter. Detailed analyses demonstrated that the observed negative effect of the GR on the activity of the gene was mediated by direct interaction between the glucocorticoid receptor and the Jun component of the AP-1 transcription complex. Work in a number of laboratories has identified a number of other instances in which direct interaction between member of the nuclear receptor family and other transcription factors is responsible for the modulation of gene activity (Uht *et al.*, 1997; Lobaccaro *et al.*, 1999).

An additional intensively studied model is the interaction between the glucocorticoid receptor and the NF- $\kappa$ B transcription factor. Glucocorticoids have long been recognized as anti-inflammatory and immunosuppressive agents, and the basis for this activity was traced to the ability of such agents to interfere with the expression of inflammatory cytokines. As many such responsive genes lack canonical glucocorticoid responsive elements, alternative mechanisms were explored, including the hormonal regulation of negative regulators of NF- $\kappa$ B activity (Auphan *et al.*, 1995; Scheinman *et al.*, 1995). Although evidence supporting such an indirect mechanism has been presented for selected cell types, the bulk of evidence at present supports a functional interaction between the RelA subunit of NF- $\kappa$ B and the DNA binding domain of the glucocorticoid receptor that results in the interference of the

transcriptional activity of both (Wissink *et al.*, 1997; Liden *et al.*, 1997). Overall, it is likely that both mechanisms play some role in the patterns of regulation observed in different cell types (Dumont *et al.*, 1998; Wissink *et al.*, 1998; McKay and Cidlowski, 1999).

Finally, in addition to the regulation of genes via transcriptional mechanisms, in some instances the steady state levels of specific mRNAs appear to be regulated by alterations in the stability of mRNA. The mechanisms by which such changes are controlled have been much less well studied. One of the most dramatic examples of this type is the regulation of vitellogenin mRNA that accumulates in male *Xenopus* liver in response to estrogen treatment. In the elegant studies of Shapiro and colleagues, large changes in the hepatic content of vitellogenin mRNA were demonstrated to reflect alterations of vitellogenin mRNA stability. Experiments employing fusion genes traced these effects to a specific segment of the vitellogenin transcript. Estrogen was found to regulate the expression of an RNA-binding protein, vigilin, which regulated the stability of the vitellogenin target (Dodson and Shapiro, 1997). Although this system affords a dramatic example of how gene expression can be regulated by steroid hormones, it is unclear whether such findings are generally applicable to the regulation of other genes in which changes of RNA stability have been observed or inferred.

In this light, however, the studies of glucocorticoid receptor knockout mice have proven quite provocative. In early experiments, Schütz and co-workers demonstrated that targeted disruption of the mouse glucocorticoid receptor gene was lethal (Cole *et al.*, 1995). In subsequent experiments, this same group modified the mouse glucocorticoid receptor such that the only GR expressed contained a targeted mutation of the DNA binding domain—A458T—that specifically disrupted the ability of the glucocorticoid receptor to form dimers and bind DNA. Surprisingly, these authors discovered that although the mutant glucocorticoid receptor expressed in these animals was not able to bind to its palindromic response element, GR activity was sufficient to permit normal viability (Reichardt *et al.*, 1998). This finding suggests the regulation of genes by the glucocorticoid receptor via modes of regulation independent of those modulated directly by dimerization/DNA binding are crucial to the survival of these animals. This finding also suggests that such modes of gene regulation may contribute substantially to the physiologic regulation of genes by the glucocorticoid receptor and potentially by other members of the nuclear receptor family as well.

## CONCLUSIONS

A great deal of insight has been gained in the last decade into the mechanisms by which nuclear receptors regulate the activities of responsive genes. Increasingly detailed structural information has become available that has further illuminated the mechanisms by which specific DNA sequences are recognized by monomeric or dimeric receptor DBDs. The determination of the structures of agonist- and antagonist-bound LBDs of several receptors has reinforced the conclusions derived from experiments focused on structural-functional relationships using the techniques of molecular biology. The identification of nuclear receptor coregulators has permitted the elucidation of important functional links between the nuclear receptors and the general transcription apparatus of the cell.

Despite this remarkable progress, a number of important challenges remain. First, although crystallographic and NMR structures have been solved for single domains of selected receptors, the precise relationships existing between the different domains of the NRs in the intact receptor proteins remain to be determined. Second is the integration of the rapidly expanding body of work surrounding the field of nuclear receptor coregulators. At this point, while a large number of proteins have been identified as interacting with members of the nuclear receptor family, the determination of the physiologic roles played by each of these proteins remains to be defined and represents a major challenge. Although daunting, this task represents one of the areas with the greatest potential yield of information that might permit the selective alteration of the expression of genes controlled by individual nuclear receptors in individual tissues or cell types.

Finally, the identification of large, stable multiprotein complexes associated with the ligand binding domains of selected nuclear receptors suggests an emerging duality. At present, it is not clear whether such complexes exist for all nuclear receptors, and if so, what role these complexes play in the regulation of responsive genes.

## REFERENCES

- Aasland, R., Stewart, A.F. and Gibson, T. (1996) The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIB. *Trends Biochem. Sci.* **21**, 87–88.
- Abraham, S.E., Lobo, S., Yaciuk, P., Wang, H.G. and Moran, E. (1993) p300, and p300-associated proteins, are components of TATA-binding protein (TBP) complexes. *Oncogene* **8**, 1639–1647.
- Alen, P., Claessens, F., Schoenmakers, E., Swinnen, J.V., Verhoeven, G., Rombauts, W. and Peeters, B. (1999a) Interaction of the putative androgen receptor-specific coactivator ARA70/ELE1 alpha with multiple steroid receptors and identification of an internally deleted ELE1beta isoform. *Mol. Endocrinol.* **13**, 117–128.
- Alen, P., Claessens, F., Verhoeven, G., Rombauts, W. and Peeters, B. (1999b) The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. *Mol. Cell Biol.* **19**, 6085–6097.
- Alland, L., Muhle, R., Hou, Jr. H., Potes, J., Chin, L., Schreiber-Agus, N. and DePinho, R.A. (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* **387**, 49–55.
- Altincicek, B., Tenbaum, S.P., Dressel, U., Thormeyer, D., Renkawitz, R. and Baniahmad, A. (2000) Interaction of the corepressor Allen with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita. *J. Biol. Chem.* **275**, 7662–7667.
- Anonymous (1999) A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97**, 161–163.
- Auphan, N., DiDonato, J.A., Rosette, C., Helmlberg, A. and Karin, M. (1995) Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B. *Science* **270**, 286–290.
- Avantaggiati, J.L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A.S. and Kelly, K. (1997) Recruitment of p300/CBP in p53-dependent signal pathways. *Cell* **89**, 1175–1184.
- Bannister, A.J. and Kouzarides, T. (1996) The CBP co-activator is a histone acetyltransferase. *Nature* **384**, 641–643.

- Baudino, T.A., Kraichely, D.M., Jefcoat, Jr. S.C., Winchester, S.K., Partridge, N.C. and MacDonald, P.N. (1998) Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. *J. Biol. Chem.* **273**, 16434–16441.
- Blumberg, B., Kang, H., Bolado, Jr. J., Chen, H., Craig, A.G., Moreno, T.A., *et al.* (1998a) BXR, an embryonic orphan nuclear receptor activated by a novel class of endogenous benzoate metabolites. *Genes Dev.* **12**, 1269–1277.
- Blumberg, B., Sabbagh, Jr. W., Juguilon, H., Bolado, Jr. J., van Meter, C.M., Ong, E.S. and Evans, R.M. (1998b) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **12**, 3195–3205.
- Bohen, S.P. and Yamamoto, K.R. (1993) Isolation of Hsp90 mutants by screening for decreased steroid receptor function. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11424–11428.
- Boudjelal, M., Wang, Z., Voorhees, J.J. and Fisher, G.J. (2000) Ubiquitin/proteasome pathway regulates levels of retinoic acid receptor gamma and retinoid X receptor alpha in human keratinocytes. *Cancer Res.* **60**, 2247–2252.
- Bourchot, B., Yaniv, M. and Muchardt, C. (1999) The activity of mammalian brm/SNF2alpha is dependent on a high-mobility-group protein I/Y-like DNA binding domain. *Mol. Cell Biol.* **19**, 3931–3939.
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H. and Moras, D. (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR- $\alpha$ . *Nature* **375**, 377–382.
- Boyes, J., Byfield, P., Nakatani, Y. and Ogryzko, V. (1998) Regulation of activity of the transcription factor GATA-1 by acetylation. *Nature* **396**, 594–598.
- Brady, M.E., Ozanne, D.M., Gaughan, L., Waite, L., Cook, S., Neal, D.E. and Robson, C.N. (1999) Tip60 is a nuclear hormone receptor coactivator. *J. Biol. Chem.* **274**, 17599–17604.
- Brou, C., Wu, J., Ali, S., Scheer, E., Lang, C., Davidson, L., Chambon, P. and Tora, L. (1993) Different TBP-associated factors are required for mediating the stimulation of transcription *in vitro* by the acidic transactivator GAL-VP16 and the two nonacidic activation functions of the estrogen receptor. *Nucl. Acids Res.* **21**, 5–12.
- Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., *et al.* (1997) Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**, 753–758.
- Burns, K., Duggan, B., Atkinson, E.A., Famulski, K.S., Nemer, M., Bleackley, R.C. and Michalak, M. (1994) Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. *Nature* **367**, 476–480.
- Caira, F., Antonson, P., Peltö-Huikko, M., Treuter, E. and Gustafsson, J.A. (2000) Cloning and characterization of RAP250, a novel nuclear receptor coactivator. *J. Biol. Chem.* **275**, 5308–5317.
- Catelli, M.G., Binart, N., Jung-Testas, I., Renoir, J.M., Baulieu, E.E., Feramisco, J.R. and Welch, W.J. (1985) The common 90-kd protein component of non-transformed “8S” steroid receptors is a heat-shock protein. *EMBOJ.* **4**, 3131–3135.
- Chakravarti, D., LaMorte, V.J., Nelson, M.C., Nakajima, T., Schulman, I.G., Juguilon, H., Montminy, M. and Evans, R.M. (1996) Role of CBP/p300 in nuclear receptor signalling. *Nature* **383**, 99–103.
- Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W. and Stallcup, M.R. (1999a) Regulation of transcription by a protein methyltransferase. *Science* **284**, 2174–2177.
- Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y. and Evans, R.M. (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms amultimeric activation complex with P.CAF and CBP/p300. *Cell* **90**, 569–580.

- Chen, H., Lin, R.J., Xie, W., Wilpitz, D. and Evans, R.M. (1999b) Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* **98**, 675–686.
- Chen, J.D. and Evans, R.M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377**, 454–457.
- Cheung, J. and Smith, D.F. (2000) Molecular Chaperone Interactions with Steroid Receptors: an Update. *Mol. Endocrinol.* **14**, 939–947.
- Chiba, H., Muramatsu, M., Nomoto, A. and Kato, H. (1994) Two human homologues of *Saccharomyces cerevisiae* SWI2/SNF2 and *Drosophila* brahma are transcriptional coactivators cooperating with the estrogen receptor and the retinoic acid receptor. *Nucl. Acids Res.* **22**, 1815–1820.
- Chiba, N., Suldan, Z., Freedman, L.P. and Parvin, J.D. (2000) Binding of liganded vitamin D receptor to the vitamin D receptor interacting protein coactivator complex induces interaction with RNA polymerase II holoenzyme. *J. Biol. Chem.* **275**, 10719–10722.
- Chrivia, J.C., Kwok, R.P.S., Lamb, N., Hagiwara, M., Montminy, M.R. and Goodman, R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855–859.
- Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., *et al.* (1995) Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev.* **9**, 1608–1621.
- Collingwood, T.N., Urnov, F.D. and Wolffe, A.P. (1999) Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *J. Mol. Endocrinol.* **23**, 255–275.
- Cordingley, M.G., Riegel, A.T. and Hager, G.L. (1987) Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary tumor virus *in vivo*. *Cell* **48**, 261–270.
- Danielsen, M., Hinck, L. and Ringold, G.M. (1989) Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* **57**, 1131–1138.
- Darimont, B.D., Wagner, R.L., Apriletti, J.W., Stallcup, M.R., Kushner, P.J., Baxter, J.D., *et al.* (1998) Structure and specificity of nuclear receptor-coactivator interactions. *Genes Dev.* **12**, 3343–3356.
- Dedhar, S., Rennie, P.S., Shago, M., Hagsteijn, C.Y., Yang, H., Filmus, J., *et al.* (1994) Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* **367**, 480–483.
- Defranco, D.B. (2000) Role of molecular chaperones in subnuclear trafficking of glucocorticoid receptors. *Kidney Int.* **57**, 1241–1249.
- Diamond, M.I., Miner, J.N., Yoshinaga, S.K. and Yamamoto, K.R. (1990) Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**, 1266–1272.
- Dittmar, K.D., Hutchison, K.A., Owens-Grillo, J.K. and Pratt, W.B. (1996) Reconstitution of the steroid receptor.hsp90 heterocomplex assembly system of rabbit reticulocyte lysate. *J. Biol. Chem.* **271**, 12833–12839.
- Dittmar, K.D. and Pratt, W.B. (1997) Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation. *J. Biol. Chem.* **272**, 13047–13054.
- Dodson, R.E. and Shapiro, D.J. (1997) Vigilin, a ubiquitous protein with 14 K homology domains, is the estrogen-inducible vitellogenin mRNA 3'-untranslated region-binding protein. *J. Biol. Chem.* **272**, 12249–12252.



- Dougherty, J.J., Puri, R.K. and Toft, D.O. (1984) Polypeptide components of two 8 S forms of chicken oviduct progesterone. *J. Biol. Chem.* **259**, 8004–8009.
- Dumont, A., Hehner, S.P., Schmitz, M.L., Gustafsson, J.A., Liden, J., Okret, S., *et al.* (1998) Cross-talk between steroids and NF-kappa B: what language? *Trends Biochem. Sci.* **23**, 233–235.
- Eckner, R., Ewen, M.E., Newsome, D., Gerdes, M., DeCaprio, J.A., Lawrence, J.B. and Livingston, D.M. (1994) Molecular cloning and functional analysis of the adenovirus El A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev.* **8**, 869–884.
- Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., *et al.* (1999) Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. *Mol. Cell Biol.* **19**, 5363–5372.
- Escriva, H., Safi, R., Hanni, C., Langlois, M.C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R. and Laudet, V. (1997) Ligand binding was acquired during evolution of nuclear receptors. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6803–6808.
- Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889–895.
- Fonsdal, K., Engedal, N., Slagsvold, T. and Saatcioglu, F. (1998) CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. *J. Biol. Chem.* **273**, 31853–31859.
- Forman, B.M., Tzamelis, I., Choi, H.S., Chen, J., Simha, D., Seol, W., Evans, R.M. and Moore, D.D. (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* **395**, 612–615.
- Freedman, L.P. and Luisi, B.F. (1993) On the mechanism of DNA binding by nuclear hormone receptors: a structural and functional perspective. *J. Cell. Biochem.* **51**, 140–150.
- Fujimoto, N., Yeh, S., Kang, H.Y., Inui, S., Chang, H.C., Mizokami, A. and Chang, C. (1999) Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. *J. Biol. Chem.* **274**, 8316–8321.
- Gewirth, D.T. and Sigler, P.B. (1995) The basis for half-site specificity explored through a non-cognate steroid receptor-DNA complex. *Nature Struct. Biol.* **2**, 386–394.
- Giguere, V. (1999) Orphan nuclear receptors: from gene to function. *Endocr. Rev.* **20**, 689–725.
- Giguere, V., Hollenberg, S.M., Rosenfeld, M.G. and Evans, R.M. (1986) Functional domains of the human glucocorticoid receptor. *Cell* **46**, 645–652.
- Glasscock, R.F. and Hoekstra, W.G. (1959) Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. *Biochem. J.* **72**, 673–682.
- Gorski, J., Toft, D., Shyamala, G., Smith, D. and Notides, A. (1968) Hormone Receptors: Studies on the interaction of Estrogen with the Uterus. *Rec. Prog. Horm. Res.* **24**, 45–80.
- Guiochon-Mantel, A., Delabre, K., Lescop, P. and Milgrom, E. (1996) The Ernst Schering Poster Award. Intracellular traffic of steroid hormone receptors. *J. Steroid Biochem. Molec. Biol.* **56**, 3–9.
- Gustafsson, J.A., Carlstedt-Duke, J., Okret, S., Wikstrom, A.C., Wrangé, O., Payvar, F. and Yamamoto, K. (1984) Structure and specific DNA binding of the rat liver glucocorticoid receptor. *J. Steroid Biochem.* **20**, 1–4.
- Gustafsson, J.A., Carlstedt-Duke, J., Poellinger, L., Okret, S., Wikstrom, A.C., Bronnegard, M., *et al.* (1987) Biochemistry, molecular biology, and physiology of the glucocorticoid receptor. *Endocr. Rev.* **8**, 185–234.
- Haataja, L., Groffen, J. and Heisterkamp, N. (1998) Identification of a novel Rac3-interacting protein C1D. *Int. J. Mol. Med.* **1**, 665–670.
- Hecht, A., Vlemminckx, K., Stemmler, M.P., van Roy, F. and Kemler, R. (2000) The p300/CBP acetyltransferases function as transcriptional coactivators of B-catenin in vertebrates. *EMBOJ.* **19**, 1839–1850.

- Heinzel, T., Lavinsky, R.M., Mullen, T.M., Soderstrom, M., Laherty, C.D., Torchia, J., Yang, W.M., *et al.* (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**, 43–48.
- Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A. Stein, R.B., Eichele, G., Evans, R.M. and Thaller, C. (1992) 9-*cis* retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**, 397–406.
- Hoock, W., Hofer, P. and Groner, B. (1992) Over-expression of the glucocorticoid receptor represses transcription from hormone responsive and non-responsive promoters. *J. Steroid Biochem. Molec. Biol.* **41**, 283–289.
- Horlein, A.J., Naar, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C.K., *et al.* (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377**, 397–404.
- Housley, P.R., Sanchez, E.R., Westphal, H.M., Beato, M. and Pratt, W.B. (1985) The molybdate-stabilized L-cell glucocorticoid receptor isolated by affinity chromatography or with a monoclonal antibody is associated with a 90–92-kDa nonsteroid-binding phosphoprotein. *J. Biol. Chem.* **260**, 13810–13817.
- Hsiao, P.W. and Chang, C. (1999) Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. *J. Biol. Chem.* **274**, 22373–22379.
- Hu, X. and Lazar, M.A. (1999) The CoNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* **402**, 93–96.
- Hu, X. and Lazar, M.A. (2000) Transcriptional repression by nuclear hormone receptors. *Trends Endocrinol. Metab.* **11**, 6–10.
- Huang, E.Y., Zhang, J., Miska, E.A., Guenther, M.G., Kouzarides, T. and Lazar, M.A. (2000) Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. *Genes Dev.* **4**, 45–54.
- Huang, S.M. and Stallcup, M.R. (2000) Mouse Zacl, a transcriptional coactivator and repressor for nuclear receptors. *Mol. Cell Biol.* **20**, 1855–1867.
- Ichinose, H., Garnier, J.M., Chambon, P. and Losson, R. (1997) Ligand-dependent interaction between the estrogen receptor and the human homologues of SWI2/SNF2. *Gene* **188**, 95–100.
- Ijpenberg, A., Jeannin, E., Wahli, W. and Desvergne, B. (1997) Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor heterodimer binding to DNA. A functional analysis of the malic enzyme gene PPAR response element. *J. Biol. Chem.* **272**, 20108–20117.
- Ikeda, M., Kawaguchi, A., Takeshita, A., Chin, W.W., Endo, T. and Onaya, T. (1999) CBP-dependent and independent enhancing activity of steroid receptor coactivator-1 in thyroid hormone receptor-mediated transactivation. *Mol. Cell. Endocrinol.* **147**, 103–112.
- Ito, M., Yuan, C.X., Malik, S., Gu, W., Fondell, J.D., Yamamura, S., Fu, Z.Y., Zhang, X., Qin, J. and Roeder, R.G. (1999) Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. *Mol. Cell.* **3**, 361–370.
- Ito, M., Yuan, C.X., Okano, H.J., Darnell, R.B. and Roeder, R.G. (2000) Involvement of the TRAP220 component of the TRAP/SMCC coactivator complex in embryonic development and thyroid hormone action. *Mol. Cell.* **5**, 683–693.
- Jackson, T.A., Richer, J.K., Bain, D.L., Takimoto, G.S., Tung, L. and Horwitz, K.B. (1997) The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. *Mol. Endocrinol.* **11**, 693–705.

- Janowski, B.A., Grogan, M.J., Jones, S.A., Wisely, G.B., Kliewer, S.A., Corey, E.J. and Mangelsdorf, D.J. (1999) Structural requirements of ligands for the oxysterol liver X receptors LXRA and LXR $\beta$ . *Proc. Natl. Acad. Sci. U.S.A.* **96**, 266–271.
- Jensen, E.V. and Jacobson, H.I. (1960) In: *Biological Activities of Steroids in Relation to Cancer*, G.Pincus and E.P.Vollmer (eds), Academic Press, New York, pp. 161–178.
- Jensen, E.V., Suzuki, T., Numata, M., Smith, S. and DeSombre, E.R. (1969) Estrogen-binding substances of target tissues. *Steroids* **13**, 417–427.
- Jenster, G., Spencer, T.E., Burcin, M.M., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1997) Steroid receptor induction of gene transcription: a two-step model. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7879–7884.
- Jenster, G., Trapman, J. and Brinkmann, A.O. (1993) Nuclear import of the human androgen receptor. *Biochem. J.* **293**, 761–768.
- Joab, I., Radanyi, C., Renou, M., Buchou, T., Catelli, M.G., Binart, N., Mester, J. and Baulieu, E.E. (1984) Common non-hormone binding component in non-transformed chick oviduct receptors of four steroid hormones. *Nature* **308**, 850–853.
- Johnson, B.A., Wilson, E.M., Li, Y., Moller, D.E., Smith, R.G. and Zhou, G. (2000) Ligand-induced stabilization of PPAR $\gamma$  monitored by NMR spectroscopy: implications for nuclear receptor activation. *J. Mol. Biol.* **298**, 187–194.
- Johnson, J.L. and Toft, D.O. (1995) Binding of p23 and hsp90 during assembly with the progesterone receptor. *Mol. Endocrinol.* **9**, 670–678.
- Jones, S.A., Moore, L.B., Shenk, J.L., Wisely, G.B., Hamilton, G.A., McKee, D.D., *et al.* (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol. Endocrinol.* **14**, 27–39.
- Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403–414.
- Kang, H.Y., Yeh, S., Fujimoto, N. and Chang, C. (1999) Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. *J. Biol. Chem.* **274**, 8570–8576.
- Kao, H.Y., Downes, M., Ordentlich, P. and Evans, R.M. (2000) Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. *Genes Dev.* **14**, 55–66.
- Kerppola, T.K., Luk, D. and Curran, T. (1993) Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity *in vitro*. *Mol. Cell. Biol.* **13**, 3782–3791.
- Kim, H.J., Kim, J.H. and Lee, J.W. (1998) Steroid receptor coactivator-1 interacts with serum response factor and coactivates serum response element-mediated transactivations. *J. Biol. Chem.* **273**, 28564–28567.
- Kim, H.J., Yi, J.Y., Sung, H.S., Moore, D.D., Jhun, B.H., Lee, Y.C. and Lee, J.W. (1999) Activating signal cointegrator 1, a novel transcription coactivator of nuclear receptors, and its cytosolic localization under conditions of serum deprivation. *Mol. Cell. Biol.* **19**, 6323–6332.
- Kim, R.H., Wang, D., Tsang, M., Martin, J., Huff, C., de Caestecker, M.P., Parks, W.T., Meng, X., Lechleider, R.J., Wang, R. and Roberts, A.B. (2000) A novel Smad nuclear interacting protein, SNIP1, suppresses P300-dependent TGF- $\beta$  signal transduction. *Genes Dev.* **14**, 1605–1616.
- Kliewer, S.A., Lehmann, J.M. and Willson, T.M. (1999a) Orphan Nuclear Receptors: shifting Endocrinology into reverse. *Science* **284**, 757–760.

- Kliwer, S.A., Lehmann, J.M., Milburn, M.V. and Willson, T.M. (1999b) The PPARs and PXR: nuclear xenobiotic receptors that define novel hormone signaling pathways. *Rec. Prog. Horm. Res.* **54**, 345–367.
- Kliwer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Watson, M.A., Jones, S., *et al.* (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**, 73–82.
- Kliwer, S.A., Umesono, K., Mangelsdorf, D.J. and Evans, R.M. (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* **355**, 446–449.
- Knutti, D., Kaul, A. and Kralli, A. (2000) A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. *Mol. Cell. Biol.* **20**, 2411–2422.
- Korzus, E., Torchia, J., Rose, D.W., Xu, L., Kurokawa, R., McInerney, E.M., Mullen, T.-M., Glass, C.K. and Rosenfeld, M.G. (1998) Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science*, **279**, 703–707.
- Kosztin, D., Bishop, T.C. and Schulten, K. (1997) Binding of the estrogen receptor to DNA: the role of waters. *Biophys. J.* **73**, 557–570.
- Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.R. and Chambon, P. (1987) Functional domains of the human estrogen receptor. *Cell* **51**, 941–951.
- Kwok, R.P., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts S.G., Green, M.R. and Goodman, R.H. (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370**, 223–226.
- Lalli, E., Ohe, K., Hindelang, C. and Sassone-Corsi, P. (2000) Orphan receptor DAX-1 is a shuttling RNA binding protein associated with polyribosomes via mRNA. *Mol. Cell. Biol.* **20**, 4910–4921.
- Lange, C.A., Shen, T. and Horwitz, K.B. (2000) Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 1032–1037.
- Lanz, R.B., McKenna, N.J., Onate, S.A., Albrecht, U., Wong, J., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1999) A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. *Cell* **97**, 17–27.
- Laudet, V. (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J. Mol. Endocrinol.* **19**, 207–226.
- Lazennec, G., Ediger, T.R., Petz, L.N., Nardulli, A.M. and Katzenellenbogen, B.S. (1997) Mechanistic aspects of estrogen receptor activation probed with constitutively active estrogen receptors: correlations with DNA and coregulator interactions and receptor conformational changes. *Mol. Endocrinol.* **11**, 1375–1386.
- Lee, S.K., Anzick, S.L., Choi, J.E., Bubendorf, L., Guan, X.Y., Jung, Y.K., *et al.* (1999) A nuclear factor, ASC-2, as a cancer-amplified transcriptional coactivator essential for ligand-dependent transactivation by nuclear receptors *in vivo*. *J. Biol. Chem.* **274**, 34283–34293.
- Lee, S.K., Kirn, H.J., Na, S.Y., Kim, T.S., Choi, H.S., Im, S.Y. and Lee, J.W. (1998) Steroid receptor coactivator-1 coactivates activating protein-1-mediated transactivations through interaction with the c-Jun and c-Fos subunits. *J. Biol. Chem.* **273**, 16651–16654.
- Lehmann, J.M., McKee, D.D., Watson, M.A., Willson, T.M., Moore, J.T. and Kliwer, S.A. (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J. Clin. Invest.* **102**, 1016–1023.
- Leo, C. and Chen, J.D. (2000) The SRC family of nuclear receptor coactivators. *Gene* **245**, 1–11.
- Leygue, E., Dotzlaw, H., Watson, P.H. and Murphy, L.C. (1999) Expression of the steroid receptor RNA activator in human breast tumors. *Cancer Res.* **59**, 4190–4193.

- Li, D., Desai-Yajnik, V., Lo, E., Schapira, M., Abagyan, R. and Samuels, H.H. (1999a) NR1F3 is a novel coactivator mediating functional specificity of nuclear hormone receptors. *Mol. Cell. Biol.* **19**, 7191–7202.
- Li, X.Y., Boudjelal, M., Xiao, J.H., Peng, Z.H., Asuru, A., Kang, S., Fisher, G.J., and Voorhees, J.J. (1999b) 1,25-Dihydroxyvitamin D<sub>3</sub> increases nuclear vitamin D<sub>3</sub> receptors by blocking ubiquitin/ proteasome-mediated degradation in human skin. *Mol. Endocrinol.* **13**, 1686–1689.
- Liden, J., Delaunay, F., Rafter, I., Gustafsson, J. and Okret, S. (1997) A new function for the C-terminal zinc finger of the glucocorticoid receptor. Repression of RelA transactivation. *J. Biol. Chem.* **272**, 21467–21472.
- Lobaccaro, J.M., Poujol, N., Terouanne, B., Georget, V., Fabre, S., Lumbroso, S. and Sultan, C. (1999) Transcriptional interferences between normal or mutant androgen receptors and the activator protein 1—dissection of the androgen receptor functional domains. *Endocrinology* **140**, 350–357.
- Lucibello, F.C., Slater, E.P., Jooss, K.U., Beato, M. and Muller, R. (1990) Mutual transrepression of Fos and the glucocorticoid receptor: involvement of a functional domain in Fos which is absent in FosB. *EMBO J.* **9**, 2827–2834.
- Ma, H., Hong, H., Huang, S.M., Irvine, R.A., Webb, P., Kushner, P.J., Coetzee, G.A. and Stallcup, M.R. (1999) Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. *Mol. Cell. Biol.* **19**, 6164–6173.
- Ma, Q. and Baldwin, K.T. (2000) 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activation and DNA binding of AhR. *J. Biol. Chem.* **275**, 8432–8438.
- Mader, S., Kumar, V., de Verneuil, H. and Chambon P. (1989) Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature* **338**, 271–274.
- Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., Hull, M.V., Lustig, K.D., Mangelsdorf, D.J. and Shan, B. (1999) Identification of a nuclear receptor for bile acids. *Science* **284**, 1362–1365.
- Mangelsdorf, D.J. and Evans, R.M. (1995) The RXR heterodimers and orphan receptors. *Cell* **83**, 841–850.
- Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**, 224–229.
- Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839.
- McKay, L.I. and Cidlowski, J.A. (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr. Rev.* **20**, 435–459.
- McKenna, N.J., Lanz, R.B. and O'Malley, B.W. (1999a) Nuclear receptor coregulators: cellular and molecular biology. *Endocr. Rev.* **20**, 321–344.
- McKenna, N.J., Xu, J., Nawaz, Z., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1999b) Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. *J. Steroid Biochem. Molec. Biol.* **69**, 3–12.
- Meinke, G. and Sigler, P.B. (1999) DNA-binding mechanism of the monomeric orphan nuclear receptor NGFI-B. *Nature Struct. Biol.* **6**, 471–477.
- Moilanen, A.M., Poukka, H., Karvonen, U., Hakli, M., Janne, O.A. and Palvimo, J.J. (1998) Identification of a novel RING finger protein as a coregulator in steroid receptor-mediated gene transcription. *Mol. Cell. Biol.* **18**, 5128–5139.

- Monden, T., Kishi, M., Hosoya, T., Satoh, T., Wondisford, F.E., Hollenberg, A.N., Yamada, M. and Mori, M. (1999) p120 acts as a specific coactivator for 9-*cis*-retinoic acid receptor (RXR) on peroxisome proliferator-activated receptor- $\gamma$ /RXR heterodimers. *Mol. Endocrinol.* **13**, 1695–1703.
- Montano, M.M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P. and Katzenellenbogen, B.S. (1999) An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6947–6952.
- Moore, L.B., Parks, D.J., Jones, S.A., Bledsoe, R.K., Consler, T.G., Stimmel, J.B., *et al.* (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J. Biol. Chem.* **275**, 15122–15127.
- Mueller, G.C., Herranen, A.M. and Jervell, K.F. (1958) Studies on the Mechanism of Action of Estrogens. *Rec. Prog. Horm. Res.* **14**, 95–139.
- Muller, J.M., Isele, U., Metzger, E., Rempel, A., Moser, M., Pscherer, A., *et al.* (2000) FHL2, a novel tissue-specific coactivator of the androgen receptor. *EMBOJ.* **19**, 359–369.
- Na, S.Y., Choi, H.S., Kim, J.W., Na, D.S. and Lee, J.W. (1998a) Bcl3, an I $\kappa$ B protein, as a novel transcription coactivator of the retinoid X receptor. *J. Biol. Chem.* **273**, 30933–30938.
- Na, S.Y., Lee, S.K., Han, S.J., Choi, H.S., Im, S.Y. and Lee, J.W. (1998b) Steroid receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor  $\kappa$ B-mediated transactivation. *J. Biol. Chem.* **273**, 10831–10834.
- Nagy, L., Kao, H.Y., Chakravarti, D., Lin, R.J., Hassig, C.A., Ayer, D.E., Schreiber, S.L. and Evans, R.M. (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**, 373–380.
- Nagy, L., Kao, H.Y., Love, J.D., Li, C., Banayo, E., Gooch, J.T., Krishna, V., Chatterjee, K., Evans, R.M. and Schwabe, J.W. (1999) Mechanism of corepressor binding and release from nuclear hormone receptors. *Genes Dev.* **13**, 3209–3216.
- Nakajima, T., Uchida, C., Anderson, S.F., Lee, C.-G., Hurwitz, J., Parvin, F.D. and Montminy, M. (1997) RNA helicase A mediates association of CBP with RNA polymerase II. *Cell* **90**, 1107–1112.
- Nawaz, Z., Lonard, D.M., Dennis, A.P., Smith, C.L. and O'Malley, B.W. (1999a) Proteasome-dependent degradation of the human estrogen receptor. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1858–1862.
- Nawaz, Z., Lonard, D.M., Smith, C.L., Lev-Lehman, E., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1999b) The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Mol. Cell. Biol.* **19**, 1182–1189.
- Nolte, R.T., Wisely, G.B., Westin, S., Cobb, J.E., Lambert, M.H., Kurokawa, R., *et al.* (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor- $\gamma$ . *Nature* **395**, 137–143.
- Norman, A.W., Adams, D., Collins, E.D., Okamura, W.H. and Fletterick, R.J. (1999) Three-dimensional model of the ligand binding domain of the nuclear receptor for 1 $\alpha$ , 25-dihydroxy-vitamin D(3). *J. Cell. Biochem.* **74**, 323–333.
- Ogryzko, V.V., Schiltz, R.L., Russanova, V., Howard, B.H. and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953–959.
- Onate, S.A., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357.
- Ordentlich, P., Downes, M., Xie, W., Genin, A., Spinner, N.B. and Evans, R.M. (1999) Unique forms of human and mouse nuclear receptor corepressor SMRT. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 2639–2644.

- Ostlund Farrants, A.K., Blomquist, P., Kwon, H. and Wrangé, O. (1997) Glucocorticoid receptor-glucocorticoid response element binding stimulates nucleosome disruption by the SWI/SNF complex. *Mol. Cell. Biol.* **17**, 895–905.
- Papoutsopoulou, S. and Janknecht, R. (2000) Phosphorylation of ETS transcription factor ER81 in a complex with its coactivators CREB-binding protein and p300. *Mol. Cell. Biol.* **20**, 7300–7310.
- Parker, M.G. (1998) Transcriptional activation by oestrogen receptors. *Biochem. Soc. Symp.* **3**, 5–50.
- Pazin, M.J. and Kadonaga, J.T. (1997) What's up and down with histone deacetylation and transcription? *Cell* **89**, 325–328.
- Perkins, N.D., Felzien, L.K., Belts, J.C., Leung, K., Beach, D.H. and Nabel, G.J. (1997) Regulation of NF- $\kappa$ B by cyclin-dependent kinases associated with the p300 coactivator. *Science* **275**, 523–527.
- Picard, D., Khursheed, B., Garabedian, M.J., Fortin, M.G., Lindquist, S. and Yamamoto, K.R. (1990a) Reduced levels of hsp90 compromise steroid receptor action *in vivo*. *Nature* **348**, 166–168.
- Picard, D., Kumar, V., Chambon, P. and Yamamoto, K.R. (1990b) Signal transduction by steroid hormones: nuclear localization is differentially regulated in estrogen and glucocorticoid receptors. *Cell Regul.* **1**, 291–299.
- Pike, A.C., Brzozowski, A.M., Hubbard, R.E., Bonn, T., Thorsell, A.G., Engstrom, O., *et al.* (1999) Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* **18**, 4608–4618.
- Poukka, H., Aarnisalo, P., Karvonen, U., Palvimo, J.J. and Janne, O.A. (1999) Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. *J. Biol. Chem.* **274**, 19441–19446.
- Poukka, H., Aarnisalo, P., Santti, H., Janne, O.A. and Palvimo, J.J. (2000) Coregulator small nuclear RING finger protein (SNURF) enhances Spl- and steroid receptor-mediated transcription by different mechanisms. *J. Biol. Chem.* **275**, 571–579.
- Pratt, W.B. and Toft, D.O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.* **18**, 306–360.
- Rachez, C. and Freedman, L.P. (2000) Mechanisms of gene regulation by vitamin D (3) receptor: a network of coactivator interactions. *Gene* **246**, 9–21.
- Rachez, C., Gamble, M., Chang, C.P., Atkins, G.B., Lazar, M.A. and Freedman, L.P. (2000) The DRIP complex and SRC-1/p160 coactivators share similar nuclear receptor binding determinants but constitute functionally distinct complexes. *Mol. Cell. Biol.* **20**, 2718–2726.
- Rachez, C., Lemon, B.D., Suldan, Z., Bromleigh, V., Gamble, M., Naar, A.M., Erdjument-Bromage, H., Tempst, P. and Freedman, L.P. (1999) Ligand-dependent transcription activation by nuclear receptors requires the DRIP complex. *Nature* **398**, 824–828.
- Rastinejad, F., Perlmann, T., Evans, R.M. and Sigler, P.B. (1995) Structural determinants of nuclear receptor assembly on DNA direct repeats. *Nature* **375**, 203–211.
- Rastinejad, F., Wagner, T., Zhao, Q. and Khorasanizadeh, S. (2000) Structure of the RXR-RAR DNA-binding complex on the retinoic acid response element DR1. *EMBO J.* **19**, 1045–1054.
- Reichardt, H.M., Kaestner, K.H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., *et al.* (1998) DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* **93**, 531–541.
- Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H. and Moras, D. (1995) Crystal structure of the RAR-ligand-binding domain bound to all-trans retinoic acid. *Nature* **378**, 681–689.

- Ribeiro, R.C., Apriletti, J., Wagner, R.L., West, B.L., Feng, W., Huber, R., *et al.* (1998) Mechanisms of thyroid hormone action: insights from X-ray crystallographic and functional studies. *Rec. Prog. Horm. Res.* **53**, 351–392.
- Robyr, D., Wolffe, A.P., and Wahli, W. (2000) Nuclear hormone receptor coregulators in action: Diversity for shared tasks. *Mol. Endocrinol.* **14**, 329–347.
- Rochel, N., Renaud, J.-P., Ruff, M., Vivat, V., Granger, F., Bonnier, D., *et al.* (1997) Purification of the human RAR gamma ligand-binding domain and crystallization of its complex with all-trans retinoic acid. *Biochem. Biophys. Res. Commun.* **230**, 293–296.
- Rochel, N., Wurtz, J.M., Mitschler, A., Klaholz, B. and Moras, D. (2000) The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand. *Mol. Cell.* **5**, 173–179.
- Rousseau, G.G. (1984) Structure and regulation of the glucocorticoid hormone receptor. *Mol. Cell. Endocrinol.* **38**, 1–11.
- Sanchez, E.R., Toft, D.O., Schlesinger, M.J. and Pratt, W.B. (1985) Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. *J. Biol. Chem.* **260**, 12398–12401.
- Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H. and Vennstrom, B. (1986) The c-erb-A protein is a high-affinity receptor for thyroid hormone. *Nature* **324**, 635–640.
- Savory, J.G., Hsu, B., Laquian, I.R., Giffin, W., Reich, T., Hache, R.J. and Lefebvre, Y.A. (1999) Discrimination between NL1- and NL2-mediated nuclear localization of the glucocorticoid receptor. *Mol. Cell. Biol.* **19**, 1025–1037.
- Scheinman, R.I., Cogswell, P.C., Lofquist, A.K. and Baldwin, Jr. A.S. (1995) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* **270**, 283–286.
- Schoenmakers, E., Verrijdt, G., Peeters, B., Verhoeven, G., Rombauts, W. and Claessens, F. (2000) Differences in DNA binding characteristics of the androgen and glucocorticoid receptors can determine hormone-specific responses. *J. Biol. Chem.* **275**, 12290–12297.
- Schule, R., Rangarajan, P., Klierer, S., Ransone, L.J., Bolado, J., Yang, N., Verma, I.M. and Evans, R.M. (1990) Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* **62**, 1217–1226.
- Schwabe, J.W., Chapman, L., Finch, J.T. and Rhodes, D. (1993) The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. *Cell* **75**, 567–578.
- Seol, W., Choi, H.S. and Moore, D.D. (1996) An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. *Science* **272**, 1336–1339.
- Shemshedini, L., Ji, J.W., Brou, C., Chambon, P. and Gronemeyer, H. (1992) *In vitro* activity of the transcription activation functions of the progesterone receptor: evidence for intermediary factors. *J. Biol. Chem.* **267**, 1834–1839.
- Shemshedini, L., Knauthe, R., Sassone-Corsi, P., Pornon, A. and Gronemeyer, H. (1991) Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors. *EMBOJ.* **10**, 3839–3849.
- Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., *et al.* (1998) The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**, 927–937.
- Smith, C.L., Nawaz, Z. and O'Malley, B.W. (1997) Coactivator & corepressor regulation of the agonist/ antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol. Endocrinol.* **11**, 657–666.



- Soderstrom, M., Vo, A., Heinzel, T., Lavinsky, R.M., Yang, W.M., Seto, E., *et al.* (1997) Differential effects of nuclear receptor corepressor (N-CoR) expression levels on retinoic acid receptor-mediated repression support the existence of dynamically regulated corepressor complexes. *Mol. Endocrinol.* **11**, 682–692.
- Sternsdorf, T., Puccetti, E., Jensen, K., Hoelzer, D., Will, H., Ottmann, O.G. and Ruthardt, M. (1999) PI C-1/SU MO-1-modified PML-retinoic acid receptor alpha mediates arsenic trioxide-induced apoptosis in acute promyelocytic leukemia. *Mol. Cell. Biol.* **19**, 5170–5178.
- Sudarsanam, P. and Winston, F. (2000) The Swi/Snf family: nucleosome-remodeling complexes and transcriptional control. *Trends Genet.* **16**, 345–350.
- Syvala, H., Vienonen, A., Zhuang, Y.H., Kivineva, M., Ylikomi, T. and Tuohimaa, P. (1998) Evidence for enhanced ubiquitin-mediated proteolysis of the chicken progesterone receptor by progesterone. *Life Sci.* **63**, 1505–1512.
- Szego, C.M. and Roberts, S. (1953) Steroid Action and Interaction in Uterine Metabolism. *Rec. Prog. Horm. Res.* **8**, 419–469.
- Takeshita, A., Yen, P.M., Misiti, S., Cardona, G.R., Liu, Y. and Chin, W.W. (1996) Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. *Endocrinology* **137**, 3594–3597.
- Tanenbaum, D.M., Wang, Y., Williams, S.P. and Sigler, P.B. (1998) Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5998–6003.
- Tasset, D., Tora, L., Fromental, C., Scheer, E. and Chambon, P. (1990) Distinct classes of transcriptional activating domains function by different mechanisms. *Cell* **62**, 1177–1187.
- Tcherepanova, I., Puigserver, P., Norris, J.D., Spiegelman, B.M. and McDonnell, D.P. (2000) Modulation of estrogen receptor-alpha transcriptional activity by the coactivator PGC-1. *J. Biol. Chem.* **275**, 16302–16308.
- Torchia, J., Rose, D.W., Inostroza, J., Kamel, Y., Westin, S., Glass, C.K. and Rosenfeld, M.G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387**, 677–684.
- Truss, M., Bartsch, J., Schelbert, A., Haché, R.J.G. and Beato, M. (1995) Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promoter *in vivo*. *EMBO J.* **14**, 1737–1751.
- Tuohimaa, P., Renoir, J.M., Radanyi, C., Mester, J., Joab, I., Buchou, T. and Baulieu, E.E. (1984) Antibodies against highly purified B-subunit of the chick oviduct progesterone receptor. *Biochem. Biophys. Res. Commun.* **119**, 433–439.
- Uht, R.M., Anderson, C.M., Webb, P. and Kushner, P.J. (1997) Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. *Endocrinology* **138**, 2900–2908.
- Umesono, K. and Evans, R.M. (1989) Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* **57**, 1139–1146.
- Umesono, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* **65**, 1255–1266.
- Vega, R.B., Huss, J.M. and Kelly, D.P. (2000) The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol. Cell. Biol.* **20**, 1868–1876.
- Verrijdt, G., Schoenmakers, E., Haelens, A., Peelers, B., Verhoeven, G., Rombauts, W. and Claessens, F. (2000) Change of specificity mutations in androgen-selective enhancers. Evidence for a role of differential DNA binding by the androgen receptor. *J. Biol. Chem.* **275**, 2298–2305.

- Wada, H., Hasegawa, K., Morimoto, T., Kakita, T., Yanazume, T. and Sasayama, S. (2000) A p300 protein as a coactivator of GATA-6 in the transcription of the smooth muscle-myosin heavy chain gene. *J. Biol. Chem.* **275**, 25330–25335.
- Wade, P.A. and Wolffe, A.P. (1999) Transcriptional regulation: switching circuitry. *Curr. Biol.* **9**, R221–224.
- Wagner, R.L., Apriletti, J.W., McGrath, M.E., Baxter, J.D. and Fletterick, R.J. (1995) A structural role for hormone in the thyroid hormone receptor. *Nature* **378**, 690–697.
- Wallberg, A.E., Neely, K.E., Hassan, A.H., Gustafsson, J.A., Workman, J.L. and Wright, A.P. (2000) Recruitment of the SWI-SNF chromatin remodeling complex as a mechanism of gene activation by the glucocorticoid receptor taul activation domain. *Mol. Cell. Biol.* **20**, 2004–2013.
- Wang, H., Chen, J., Hollister, K., Sowers, L.C. and Forman, B.M. (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol. Cell.* **3**, 543–553.
- Webb, P., Lopez, G.N., Greene, G.L., Baxter, J.D. and Kushner, P.J. (1992) The limits of the cellular capacity to mediate an estrogen response. *Mol. Endocrinol.* **6**, 157–167.
- Weinberger, C., Thompson, C.C., Ong, E.S., Lebo, R., Gruol, D.J. and Evans, R.M. (1986) The c-erb-A gene encodes a thyroid hormone receptor. *Nature* **324**, 641–646.
- Weiss, R.E., Xu, J., Ning, G., Pohlenz, J., O'Malley, B.W. and Refetoff, S. (1999) Mice deficient in the steroid receptor co-activator 1 (SRC-1) are resistant to thyroid hormone. *EMBO J.* **18**, 1900–1904.
- Wheeler, D.G., Horsford, J., Michalak, M., White, J.H. and Hendy, G.N. (1995) Calreticulin inhibits vitamin D3 signal transduction. *Nucl. Acids Res.* **23**, 3268–3274.
- Wiesenberg, I., Chiesi, M., Missbach, M., Spanka, C., Pignat, W. and Carlberg, C. (1998) Specific activation of the nuclear receptors PPARgamma and RORA by the antidiabetic thiazolidinedione BRL 49653 and the antiarthritic thiazolidinedione derivative CGP 52608. *Mol. Pharmacol.* **53**, 1131–1138.
- Williams, S.P. and Sigler, P.B. (1998) Atomic structure of progesterone complexed with its receptor. *Nature* **393**, 392–396.
- Wissink, S., van Heerde, E.G., Schmitz, M.L., Kalkhoven, E., van der Burg, B., Baeuerle, P.A. and van der Saag, P.T. (1997) Distinct domains of the RelA NF-kappaB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. *J. Biol. Chem.* **272**, 22278–22284.
- Wissink, S., van Heerde, E.G., van der Burg, B. and van der Saag, P.T. (1998) A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. *Mol. Endocrinol.* **12**, 355–363.
- Wolffe, A.P. and Pruss, D. (1996) Targeting chromatin disruption: Transcription regulators that acetylate histones. *Cell* **84**, 817–819.
- Wurtz, J.M., Egner, U., Heinrich, N., Moras, D. and Mueller-Fahrnow, A. (1998) Three-dimensional models of estrogen receptor ligand binding domain complexes, based on related crystal structures and mutational and structure-activity relationship data. *J. Med. Chem.* **41**, 1803–1814.
- Xu, J., Liao, L., Ning, G., Yoshida-Komiya, H., Deng, C. and O'Malley, B.W. (2000) The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6379–6384.
- Xu, J., Qiu, Y., DeMayo, F.J., Tsai, S.Y., Tsai, M.-J. and O'Malley, B.W. (1998) Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* **279**, 1922–1925.
- Xu, L., Glass, C.K. and Rosenfeld, M.G. (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Devel.* **9**, 140–147.

- Yang, X.J., Ogryzko, V.V., Nishikawa, J., Howard, B.H. and Nakatani, Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein El A. *Nature* **382**, 319–324.
- Yang-Yen, H.F., Chambard, J.C., Sun, Y.L., Smeal, T., Schmidt, T.J., Drouin, J. and Karin, M. (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* **62**, 1205–1215.
- Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P., Wang, C., Su, C. and Chang, C. (1998) Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. *Biochem. Biophys. Res. Commun.* **248**, 361–367.
- Ylikomi, T., Bocquel, M.T., Berry, M., Gronemeyer, H. and Chambon, P. (1992) Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.* **11**, 3681–3694.
- Zamir, I., Dawson, J. Lavinsky, R.M., Glass, C.K., Rosenfeld, M.G. and Lazar, M.A. (1997) Cloning and characterization of a corepressor and potential component of the nuclear hormone receptor repression complex. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14400–14405.
- Zanaria, E., Muscatelli, i F., Bardoni, B., Strom, T.M., Guioli, S., Guo, W., *et al.* (1994) An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. *Nature* **372**, 635–641.
- Zhao, Q., Chasse, S.A., Devarakonda, S., Sierk, M.L., Ahvazi, B. and Rastinejad, F. (2000) Structural basis of RXR-DNA interactions. *J. Mol. Biol.* **296**, 509–520.
- Zhou, Z.X., Sar, M., Simental, J.A., Lane, M.V. and Wilson, E.M. (1994) A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNA-binding domain and modulation by NH<sub>2</sub>-terminal and carboxyl-terminal sequences. *J. Biol. Chem.* **269**, 13115–13123.
- Zhu, Y., Kan, L., Qi, C., Kanwar, Y.S., Yeldandi, A.V., Rao, M.S. and Reddy, J.K. (2000) Isolation and characterization of peroxisome proliferator-activated receptor (PPAR) interacting protein (PRIP) as a coactivator for PPAR. *J. Biol. Chem.* **275**, 13510–13516.

# 15.

## NEUROSTEROIDS AND BRAIN STEROLS

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Conventionally, steroids operate via transcription, but a subclass of brain-active steroids, dubbed neurosteroids, may govern cognitive processes via membrane-associated receptors. *De novo* synthesis of neurosteroids within the brain has been discussed; we suggest that these may derive primarily from the circulation. In contrast, the brain is largely self-sufficient in cholesterol. Synthesis and metabolism of cholesterol and its oxysterol derivatives appears to be crucial to brain development and function, emphasized by drugs (anti-convulsants, neuroleptics) and mutations (Smith-Lemli-Opitz, Niemann-Pick disease type C, cerebrotendinous xanthomatosis) that affect these pathways and have marked brain effects. Receptors for steroids and sterols are discussed, particularly those at cell-surface and intracellular membranes including sites of sterol metabolism and trafficking (including sigma-1, the emopamil binding protein [EBP], and the peripheral benzodiazepine receptor [PBR]). Potential overlaps between sterol and steroid signaling are discussed. In addition to regulating neuronal activity, we suggest that steroids and sterols may regulate proliferative and degenerative processes in the brain including apoptosis induction. Evidence is presented for cross-talk between activity of neurotransmitter receptors at the cell surface (e.g., GABA<sub>A</sub>) and pathways operating within the cell; local sterol signaling could potentially extend between cells. We also address whether changes in neurosteroid signaling mediated by the adrenal steroid dehydroepiandrosterone (DHEA) could contribute to age-related cognitive impairments.

Definitions: Cerebrosterol, cholest-5-ene-3 $\beta$ ,24(S)-diol [24(S)-hydroxycholesterol]; DHEA, dehydroepiandrosterone; Oxysterol, hydroxylated derivatives of cholesterol (or dehydrocholesterol).

KEY WORDS: brain, hormone, metabolism, neurosteroid, steroid, synthesis.

### INTRODUCTION

Signaling by steroids and sterols is widespread in vertebrates, insects, plants and fungi; the mammalian brain is no exception. In vertebrates, steroids produced from peripheral

endocrine organs, including the adrenal, gonads and placenta, govern a diversity of physiological parameters including information processing in the brain. It has been suggested that steroid synthesis and metabolism take place in the CNS; brain-derived “neurosteroids” may play a role in cognitive processes. However, although brain function is modulated by steroids (and in turn the brain can govern peripheral (endocrine) steroid hormone production), what is unusual about the brain is its particular dependence on steroid/sterol synthesis and metabolism—deficits in cholesterol metabolism can be catastrophic for cognition while other brain and body systems continue to operate. Here we do not attempt to provide a comprehensive overview of brain steroid metabolism and action; the interested reader is referred to one of the excellent recent reviews that address specific topics (Baulieu, 1997, 1998; de Kloet *et al.*, 1998; McEwen and Alves, 1999; Rupprecht and Holsboer, 1999; Mensah-Nyagan *et al.*, 1999). Neither do we focus on well-trodden areas of neurotransmitter receptor targets, plasma cholesterol transport, and sulfation/desulfation of steroids. We have chosen to emphasize developmental and maintenance processes rather than acute modulation and, in particular, we have sought to emphasize new directions of exploration rather than revisit old territory. In the following we commence by examining the paradigm of estrogen-dependent masculinization of brain function. We then consider evidence for local synthesis and metabolism of cholesterol and steroids, and overview the targets, some unusual, that mediate their action. We continue by discussing the emerging contention that sterol metabolism is pivotal to brain function and dwell on the role of neurosterols and neurosteroids in apoptotic and developmental processes in the mammalian brain. We conclude with a list of observations that emphasise fresh avenues of research.

### STEROIDS GOVERN BRAIN DEVELOPMENT AND FUNCTION

Like other tissues, the brain responds to steroids, both locally produced and derived from the endocrine organs. This is exemplified by the masculinization of behavior produced by exposure to perinatal steroids (reviewed in [Box 15.1](#)). Perinatally, 17 $\beta$ -estradiol (estrogen) enters the brain only poorly. In males, gonadal testosterone (which enters the brain more freely) is converted to 17 $\beta$ -estradiol by aromatase ([Box 15.2](#)); estradiol then elicits male-type development and behavior. Conversely, estradiol in the adult (which does enter the brain) fails to cause male-type behavior. These observations highlight two important aspects of steroid regulation. First, steroid action is modified according to local delivery and metabolism in the target tissue. Second, steroids can have separate effects during development and in the adult brain. While the details of steroid imprinting of gender-specific behavior remain to be fully elucidated, an understanding of the effects of sex steroids on the brain is of great importance, particularly in view of the undoubted role that estrogens and related molecules play in modulating neuronal function in the adult brain and their potential for the therapy of neurodegenerative diseases including Alzheimer's (McEwen and Alves, 1999; Toran-Allerand *et al.*, 1999).

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**BOX 15.1****SEX STEROID MASCULINIZATION OF BRAIN FUNCTION**

The brains of males and females display significant structural differences, particularly in the sexually dimorphic nucleus of the medial preoptic area, but also in the base nucleus of the stria terminalis, the vomeronasal organ, hippocampus, amygdala and hypothalamus (Cooke *et al.*, 1998). Sexual behavior in most mammalian species is also dimorphic, though the correlation between structural and behavioral differences is unknown. Female rodents, in the presence of a male, display lordosis (i.e., adopt a characteristic posture that facilitates mating). The predisposition to the lordotic response is determined by two factors. First, by the absence of exposure to masculinizing androgens in the perinatal period; treatment of females with testosterone at this time impairs lordosis and leads to male-type behavior. Second, by the continued production of female sex steroids including estradiol and progesterone. Castration of male neonates produces animals that display lordosis when suitably primed with female hormones; normal males fail to show this behavior.

Perhaps counter-intuitively, the hormone responsible for masculinization of the brain and behavior is the female sex steroid estrogen ( $17\beta$ -estradiol), and defeminization can occur in the absence of a functional androgen receptor (Olsen, 1979). According to the paradigm for steroid masculinization of brain, maternal gonadal estradiol cannot enter the brain because it binds with high affinity to alpha-fetoprotein, a steroid-binding protein produced by the fetal liver (but not by the adult). However, brain estradiol can be produced, *in situ*, by enzymatic aromatization of testosterone derived from the bloodstream, mediated by brain aromatase enzyme (Box 15.2). Unlike estradiol, testosterone fails to bind to alpha-fetoprotein, and thus can cross into the brain. By this mechanism, gonadal testosterone reaches the brain in males and not in females, but only following conversion to estradiol produces masculinization of brain development and later life behavior (MacLusky and Naftolin, 1985).

In support, prenatal treatment of rats with an aromatase inhibitor can prevent masculinization (see, for instance, Clemens and Gladue, 1978) while *in utero* transfer of testosterone from male fetuses to female sibs via the bloodstream can elicit partial masculinization of the females (Houtsmuller and Slob, 1990). The unaromatizable derivative of testosterone,  $5\alpha$ -dihydrotestosterone (DHT) is rather ineffective at inducing brain masculinization despite playing a predominant role in the development of masculine body characteristics. The sexually dimorphic nucleus (SDN) of the preoptic area is still dimorphic in rats with testicular feminization due to androgen receptor deficit (C.D.Jacobson, cited in Cooke *et al.*, 1998), demonstrating that androgen receptor activation is not essential for sex-specific brain development. Mice lacking a functional estrogen receptor alpha ( $ER\alpha$ ) have clear impairments in male-type behavior (Wersinger *et al.*, 1997; Ogawa *et al.*, 1998), although female behavior is also disrupted. Testosterone, and its estradiol metabolite, appear to cause masculinization in part by inhibiting apoptotic mechanisms associated with sex-specific neuronal loss, for instance in the SDN. They can also have direct neurotropic effects (Davis *et al.*, 1996).

However, the story is becoming more complicated—estradiol and its derivatives appear not to be the only natural steroids that affect masculinization. Although Honda *et al.* (1998) report selective deficits in male-type behavior in mice harboring a disruption of a brain promoter for the aromatase gene, in other studies young male mice lacking the

aromatase enzyme were found to be capable of breeding and displayed normal mounting behavior (Fisher *et al.*, 1998; Robertson *et al.*, 1999) although these animals became physiologically infertile as adults due to testicular abnormalities. This finding argues that aromatase-mediated conversion of testosterone, to estradiol is not essential for the development of male-type behavior, and suggests that unaromatized steroids can contribute. More recent studies have shown that aromatase gene expression is regulated by androgens (see later). Furthermore, both estradiol and testosterone may be further metabolized in brain to active hormones. Estradiol may be hydroxylated in brain to generate hormonally-active catechol estrogens by hydroxylation at the 2 (or 4) position (Fishman and Norton, 1975), possibly by another enzyme or by additional aromatase-mediated metabolism (Osawa *et al.*, 1993). Testosterone is potentially converted to dihydrotestosterone (DHT) in brain by 5 $\alpha$ -reductase and thence to downstream metabolites including the product of 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) action on DHT, 5 $\alpha$ -androstane-3 $\alpha$ , 17 $\beta$ -diol (see below and [Figure 15.1](#)); although not demonstrated, such steroids could cause masculinization independently of the androgen receptor. It is also likely that androgens and estrogens play multiple roles in the developing brain, and interact with diverse nuclear and cell-surface receptors activating a variety of downstream signal-transduction pathways (Beyer, 1999; McEwen and Alves, 1999; Toran-Allerand, 1999).

## **BOX 15.2**

### **AROMATASE**

This enzyme is responsible for the conversion of testosterone to estradiol and ensuing brain masculinization subsequent to specific activation of estrogen receptors. The same enzyme catalyzes the conversion of androstenedione to estrone, a major pathway for estrogenic steroid production subsequent to the menopause. In rodents and in human a single gene appears to be responsible for CYP19 (P450arom) activity, though the existence of additional GYP 19 genes in pig and goldfish (Choi *et al.*, 1997; Tchoudakova and Callard, 1998) argues that additional rodent and human genes may remain to be uncovered. CYP19 is somewhat unusual among the cytochrome P450s because CYP19 expression derives from several different upstream promoter regions. In all cases examined, however, the encoded protein is identical (Simpson *et al.*, 1997). A major brain-specific promoter is utilized in the central nervous system (Honda *et al.*, 1994) although another upstream promoter, specific to cortex, has also been reported (Kato *et al.*, 1997). As expected for a key hormonal activation step, enzyme production is subject to a number of regulatory controls. Aromatase expression, principally in neurons, is high during pre- and peri-natal development, particularly in hypothalamic, limbic regions and developing sensory centers (Horvath and Wikler, 1999), but declines in adulthood (reviewed by Lephart, 1997). Although brain masculinization reflects conversion of gonadal testosterone to estradiol, emerging evidence suggests that CYP19 expression is itself regulated by androgens as well as estrogens (Hutchinson *et al.*, 1997), arguing that androgen and estrogen act in concert to produce brain masculinization. Aromatase enzyme activity is also modulated by natural breakdown products of testosterone and by environmental influences (reviewed by Hutchison, 1993). Brain aromatase is also reduced by prenatal stress (Jimbo *et al.*, 1998); this could, plausibly, predispose to feminization in some species.

In addition to local metabolism (activation/inactivation) in the central nervous system, the brain has been suggested to be a site of *de novo* steroid synthesis, giving rise to local production of “neurosteroids”. We next address the evidence that the ubiquitous steroid precursor, cholesterol (see [Figure 15.1](#)), is produced locally in brain.

### CHOLESTEROL SYNTHESIS IN BRAIN

Cholesterol is a major component (~30–80%) of cell membranes, including those in the brain. In rats the major phase of brain membrane formation takes place during the perinatal period, during which many neurons become myelinated. Even during this period of peak demand, local synthesis seems to fulfil the cholesterol requirements of the brain. *In vivo*, cholesterol is rapidly produced in the brain from radiolabeled precursors (Sérourne and Chevallier, 1976) while, *in vitro*, primary cultures from newborn rat forebrain, consisting primarily of astrocytes and oligodendrocytes, convert [3H]-mevinolactone to cholesterol; here up to 10% of applied radioactivity could be recovered as cholesterol (Hu *et al.*, 1989; Jung-Testas *et al.*, 1989), while lesser amounts of pregnenolone and 20-hydroxypregnenolone (and their esters) were also produced. Local synthesis normally satisfies brain cholesterol demand in the brain and in the peripheral nervous system, both during development and during nerve regeneration (Jurevics and Morell, 1995; Morell and Jurevics, 1996; Jurevics *et al.*, 1998).

Key cholesterol-synthesizing enzymes are expressed in brain. Cholesterol is produced from elementary precursors in an extensive series of reactions. The first and primary rate-limiting step is the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid by the enzyme hydroxy-methylglutaryl CoA reductase (HMGCR). In brain, HMGCR activity is abundant, particularly during development (Sudjic and Booth, 1976; Ness *et al.*, 1979), peaking at postnatal day 4 in the rat (Ness, 1994) but continuing to be expressed during adulthood. In mice transgenic for an HMGCR fusion gene (Mehtali, 1988; Mehtali *et al.*, 1990) reporter activity was highest in brain and was predominantly associated with oligodendrocytes in the adult (Duhamel-Clerin *et al.*, 1994). Cholesterol production is subject to end-product repression, but while liver HMGCR transcription was repressed by cholesterol and induced by blockade of cholesterol synthesis, neither endogenous HMGCR gene expression nor that of the fusion transgene responded to manipulations of peripheral cholesterol demand (Mehtali, 1988; Mehtali and Lathe, unpublished data). Thus cholesterol production in brain is regulated independently of other tissues including liver.

While the brain can satisfy the majority of its cholesterol demand, peripheral cholesterol can enter the brain (Spohn and Davison, 1972; Sérourne and Chevallier, 1976), and slow exchange between brain and plasma cholesterol is likely. In the adult, mature neurons downregulate cholesterol synthesis and an increasing proportion of brain cholesterol may be supplied by uptake of cholesterol via the apolipoprotein E (ApoE)/low density lipoprotein (LDL) receptor system (Poirier *et al.*, 1993; Poirier and Sevigny, 1998). Defective



cholesterol synthesis due to  $\Delta^7$ -reductase deficiency (Smith-Lemli-Opitz syndrome, mental retardation, developmental deficits and adrenal insufficiency) can respond to dietary cholesterol supplementation (Opitz and de la Cruz, 1994; Nowaczyk *et al.*, 1999) and, though the response is sometimes relatively weak, this may demonstrate that some cholesterol can enter the brain from the circulation, at least in adults. LDL receptor expression is found on cells of the blood-brain barrier, suggesting an involvement in cholesterol exchange (Meresse *et al.*, 1989; Dehouck *et al.*, 1998).

Moreover, the brain is often a net exporter of cholesterol (Björkhem *et al.*, 1997), and takes advantage of a unique 24(S) hydroxylation system operating in parallel with the high-density lipoprotein (HDL) export machinery (Björkhem *et al.*, 1999). The 24(S) hydroxylase enzyme, CYP46, is primarily expressed in brain, with lesser amounts in adrenal (Lund *et al.*, 1999; Björkhem *et al.*, 1999).

### ARE STEROIDS SYNTHESIZED *DE NOVO* IN BRAIN?

What is the evidence that steroids are synthesized in the CNS? Though there is compelling evidence for local brain synthesis of -cholesterol, and for steroid modification (exemplified by aromatization of testosterone to estradiol), data supporting *de novo* synthesis of pregnenolone and derivative steroids are more contentious. Steroids differ from sterols, such as cholesterol, in that the long hydrophobic side-chain is lost (see Figure 15.1), boosting both solubility and signaling potential. Side-chain cleavage (sec) can occur chemically, but *in vivo* is catalyzed by the enzyme P450<sub>sc</sub> (CYP11A1), converting cholesterol (and side-chain hydroxylated cholesterols) to pregnenolone, for onward conversion to generate DHEA, progesterone and derivative steroids. Evidence for brain steroidogenesis derives from four sources. First, that the brain naturally contains significant concentrations of steroids. Second, molecular data suggesting that key steroidogenic enzymes are expressed in brain. Third, from biochemical studies that address steroid conversions in brain. Fourth, that brain steroid levels may not be entirely subservient to peripheral plasma concentrations.

### Steroids are present in brain

The first line of argument is that the brain naturally contains significant concentrations of steroids, particularly the adrenal androgens DHEA and pregnenolone. These are present in blood and brain principally in the form of sulfates and fatty acid esters; free steroid is present at much lower concentrations (Table 15.1). Primates are unusual in that DHEA (and pregnenolone) sulfates circulate in blood at strikingly high levels compared with other species. Primate DHEA levels in the circulation usually exceed pregnenolone levels by a factor of 10 (though 17-hydroxypregnenolone is a major component in some species), and effective plasma levels of DHEA sulfate of up to 1–2  $\mu$ M have been recorded. In human sciatic nerve, DHEAS remains the major form (in the order of 100 ng/g; Morfin *et al.*, 1992).

**Table 15.1** Consensus concentrations (ng/g) of neurosteroids in blood and brain<sup>1</sup>.

		<i>D</i> <sup>2,3</sup>	<i>DS</i>	<i>DL</i>	<i>P3</i>	<i>PS</i>	<i>PL</i>
HUMAN	Blood	10	2000	na	2	100	na
	Sciatic nerve	na	150	na	60	25	30
	CSF	0.2	1	na	na	na	na
RAT	Blood	0.1	2	na	20	10	na
	Brain	3	2	30	20	10	70

Concentrations are compiled from published values for human blood (see Aso, 1976; Orentreich *et al.*, 1984, 1992; Guazzo *et al.*, 1996), human sciatic nerve (Morfin *et al.*, 1992), human cerebrospinal fluid (Schwarz and Pohl, 1992; Guazzo *et al.*, 1996) and rat plasma and brain (Corpechot *et al.*, 1981, 1983, 1985; Jo *et al.*, 1989). Multiple values have been averaged across different reports. D, free DHEA; DS, DHEA sulfate; DL, lipoidal/fatty acid esters of DHEA; P, free pregnenolone; PS, pregnenolone sulfate; PL, lipoidal/fatty acid esters of pregnenolone. na, values not available.

Notes:

1. Concentrations were generally obtained by radioimmunoassay; values obtained by mass-spectroscopy may be 100 to 1000-fold lower (Uzunova *et al.*, 1998; Kim *et al.*, 2000), for reasons that are not understood.

2. In human, concentrations of DHEA and its sulfate decline with age and differ between males and females (Orentreich *et al.*, 1984, 1992). Concentrations can show marked diurnal variations (Robel *et al.*, 1986) and active responses; hypothalamic but not amygdala or olfactory bulb DHEA levels were boosted 6-fold by exposure of male rats exposed to females (Robel *et al.*, 1986).

3. Apparent brain concentrations of P and D can be increased up to 7-fold by pretreatment of extracts with organic base and FeSO<sub>4</sub> (Prasad *et al.*, 1994).

while the concentration of free pregnenolone can exceed the concentrations of its sulfate (~60ng/g versus ~25ng/g) with a significant contribution of fatty acid esters (~30ng/g) (Morfin *et al.*, 1992).

In rodent blood DHEA and pregnenolone levels are much lower, and pregnenolone is the major steroid, exceeding DHEA levels by 20-fold or more (Robel *et al.*, 1987). Again the sulfates predominate (Table 15.1). In rat brain, both steroids are present (Corpechot *et al.*, 1981, 1983) but the concentration of pregnenolone (20–40ng/g, equivalent to around 0.1μM) was only marginally above that detected in kidney or spleen (9–15ng/g) and substantially below the level in adrenal (5300ng/g; Robel *et al.*, 1987). In the study of Korneyev *et al.* (1993), steroid concentrations were highest in olfactory bulb (10–14ng/g, equivalent to around 30nM) but slightly lower in other brain regions (4–7ng/g). Prasad *et al.* (1994) suggest that the apparent rat brain concentrations of pregnenolone and DHEA may be boosted markedly (to ~450ng/g; pregnenolone) by tissue treatment with organic bases or the reducing agent FeSO<sub>4</sub>, suggesting that higher concentrations of these molecules (or intermediates in their synthesis) may be present, *in vivo*, as hydroperoxides. This result remains to be confirmed.

Although DHEA levels are markedly elevated in primates compared to rodents, it is of particular note that the overall concentrations of DHEA (free+sulfate) in rat brain (~5ng/g, excluding esters) are not dissimilar from the concentrations recorded in human CSF (~1–

2ng/g, excluding esters), though substantially below those in human sciatic nerve (~150ng/g, Table 15.1).

### Molecular evidence for steroidogenic enzymes in brain

A second line of argument is that many steroidogenic enzymes, including the key cholesterol side-chain cleavage enzyme (P450<sub>scc</sub>, CYP11A1), are expressed in brain. However, these data can be difficult to interpret because some techniques are extremely sensitive (e.g., RT-PCR) and can detect trivial levels of mRNA expression. Le Goascogne *et al.* (1987) reported widespread detection of P450<sub>scc</sub> immunoreactivity in rat brain. Stromstedt and Waterman (1995) used RT-PCR to detect P450<sub>scc</sub> mRNA in all rat brain regions, but not in mouse brain. *In situ* hybridization detected P450<sub>scc</sub> mRNA in rat cortex, hippocampus, olfactory bulb and cerebellar granule, and also purkinje cells (Furukawa *et al.*, 1998), with astrocytes, oligodendrocytes and neurons all expressing mRNA as assessed by RT-PCR (Zwain and Yen, 1999a). Generally, the level of expression seems to be far below that detected in classic steroid-synthesizing tissues such as the adrenals, gonads and placenta (Sanne and Krueger, 1995; Compagnone *et al.*, 1995a, b; Kohchi *et al.*, 1998).

The critical co-enzymes adrenodoxin and P450 reductase are detected throughout the brain (Oftensbro *et al.*, 1979; Stromstedt and Waterman, 1995), while the sterol carrier protein SCP-2, that also participates in cholesterol synthesis and transport (e.g., Scallen *et al.*, 1985) is well expressed in the central nervous system (Myers-Payne *et al.*, 1996). The potential transport proteins, OPP1–6, related to the oxysterol binding proteins (OSBP) are widely expressed in the CNS (Laitinen *et al.*, 1999). Furukawa *et al.* (1998) also report *in situ* detection of mRNA encoding the intracellular cholesterol transport protein StAR, required for efficient cholesterol delivery to P450<sub>scc</sub> and steroid synthesis in adrenal, and suggest that StAR and P450<sub>scc</sub> (CYP11A1) are co-expressed in many brain regions. The brain and placenta homolog of StAR, MLN64, is also present (Watari *et al.*, 1997). On the other hand, expression of steroidogenic factor 1 (SF1), a transcription factor required in other tissues for efficient expression of steroidogenic enzymes, is detectable by RT-PCR but does not appear to be abundant in rodent brain (see Stromstedt and Waterman, 1995). mRNA is rather more robustly and widely expressed in human brain, as assessed by Northern blotting and *in situ* hybridization, but again the levels are very much lower than those in conventional steroidogenic tissues (Ramayya *et al.*, 1997). However, the dependency of brain steroidogenic enzyme production on SF-1 expression has not been demonstrated; SF-1 is not necessary for P450<sub>scc</sub> expression in developing mouse gut (Keeney *et al.*, 1995).

The situation with CYP17(17 $\alpha$ -hydroxylase/c 17,20-lyase), the enzyme that converts pregnenolone to DHEA, is not dissimilar—brain levels are low (Stromstedt and Waterman, 1995; Compagnone *et al.*, 1995a; Kohchi *et al.*, 1998) or undetectable (Le Goascogne *et al.*, 1991). Here, however, it has been suggested that the brain may be able to convert pregnenolone to DHEA by a CYP17-independent pathway (Cascio *et al.*, 1998); low levels of CYP17 might not preclude DHEA production *in vivo* from pregnenolone, or even, conceivably, by chemical oxidation of cholesterol (Prasad *et al.*, 1994).

This summary above is not exhaustive; further overviews of key enzyme pathways are provided in [Boxes 15.1–15.6](#) and in focused reviews elsewhere in this volume. In general, the brain expresses a surprising diversity of steroid-metabolizing enzymes, as revealed by biochemical conversions in brain extracts and enzymes detected by immunohistochemistry and molecular analysis of mRNA expression. [Boxes 15.2–15.6](#) separately review the different conversions, focusing on aromatase ([Box 15.2](#)), 3 $\beta$ -HSD ([Box 15.3](#)), 3 $\alpha$ -dehydrogenation and 5 $\alpha$ -reduction ([Box 15.4](#)), 11-position modifications, particularly of the glucocorticoids ([Box 15.5](#); see also chapters 7 and 12), and steroid 6 and 7 hydroxylation ([Box 15.6](#)).

### BOX 15.3

#### 3 $\beta$ -HYDROXYSTEROID DEHYDROGENASE (3 $\beta$ -HSD)

The conversion of pregnenolone to progesterone by brain (Zhao *et al.*, 1991) and by peripheral Schwann cells (Koenig *et al.*, 1995) argues that this enzyme is active *in vivo*. The brain is far from unusual though—more than 10 different tissues can catalyse this conversion (Zhao *et al.*, 1991). 3 $\beta$ -HSD both reduces the 3 $\beta$ -hydroxy group and isomerizes the 5–6 unsaturated bond (to the 4–5 position; [Figure 15.1](#)); under some circumstances the reverse reaction may predominate. 3 $\beta$ -HSD converts pregnenolone to progesterone, but also metabolizes DHEA (produced by CYP17-conversion of pregnenolone; [Figure 15.1](#)) to androstenedione. These are important steroids in their own right, and are also precursors to other steroids including testosterone and estradiol (from androstenedione), the glucocorticoids and mineralocorticoids (from progesterone) and the 3 $\alpha$ -hydroxy anesthetic steroids derived from them.

At least 6 different isoforms of 3 $\beta$ -HSD are known, in rodents (two in humans), but whereas types I, II and III catalyze progesterone and androstenedione synthesis, types IV and probably V catalyze an oxosteroid reductase reaction, reducing the 3-oxo group (Clarke *et al.*, 1993), and convert, e.g., the 3-oxo steroid testosterone to the non-androgenic (but potentially hormonally-active) steroid 5 $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol (reviewed by Simard *et al.*, 1995; Mason *et al.*, 1997; Payne *et al.*, 1997). In brain, oxidation seems to predominate (enzyme types I to III), and brain extracts metabolize pregnenolone and DHEA to progesterone and androstenedione (Zhao *et al.*, 1991; Guennoun *et al.*, 1997). Guennoun *et al.* (1995), using a probe to a conserved region for *in situ* hybridization to rat brain, reported mRNA in cerebellum, cortex, striatum, thalamus, hypothalamus, olfactory bulb, and hippocampus. Sanne and Kreuger (1995) confirmed 3 $\beta$ -HSD expression throughout rat brain, and detected 3 $\beta$ -HSDs type I, II and III but not IV mRNA by PCR. In this study expression levels were generally reported to be low, although Ukena *et al.* (1999) suggest that 3 $\beta$ -HSD expression is age-dependent, being highest in the perinatal rat cerebellum.

### Steroidogenesis in brain extracts

Third, steroid synthesis and metabolism in brain tissue extracts is well-documented. Newborn rat glia and purified mitochondria from young rats can produce pregnenolone from

cholesterol or its precursors (Hu *et al.*, 1987; Hu *et al.*, 1989; Jung-Testas *et al.*, 1989). Pregnenolone production by rat brain mitochondria is stimulated by ligands of the peripheral benzodiazepine receptor (Romeo *et al.*, 1993; McCauley, 1995) that facilitates intracellular cholesterol transport; compounds activating cAMP production also increased pregnenolone formation in retina (Guarneri *et al.*, 1994).

Onward metabolism is also reported, and in the above studies 20-hydroxypregnenolone (5-pregnen-3 $\beta$ , 20 $\alpha$ -diol), and 5-pregnen-3,20-dione were detected (Hu *et al.*, 1987, 1989) while neurons from 17-day mouse embryos only formed 20-hydroxypregnenolone (Akwa *et al.*, 1991). Here DHEA synthesis was not observed. However, although incubation of primary newborn rat forebrain cultures (primarily astrocytes and oligodendrocytes) with [3H]-pregnenolone led to the production of radiolabelled progesterone and 20-hydroxypregnenolone (Jung-Testas *et al.*, 1989), the efficiency of conversion was low and progesterone represented only 3% of applied radioactivity after 24 hours incubation. Because the primary cultures were maintained *in vitro* for 3 weeks prior to assay, they may not accurately reflect the *in vivo* situation. However, 20-hydroxyprogesterone was also the predominant metabolite of progesterone in rabbit cornea (Navarro-Ruiz *et al.*, 1987) and in whole brain extract (Carey *et al.*, 1994) though a lesser amount of 5 $\alpha$ -pregnane-3,20-dione was also produced. Kabbadj *et al.* (1993) report that 20-hydroxypregnenolone is a major product in isolated neuronal cultures, while astrocytes converted pregnenolone to 5 $\alpha$ -dihydroprogesterone and 3 $\alpha$ ,5 $\alpha$ -tetrahydroprogesterone (THPROG). Steroidogenesis has also been reported in isolated rat retina where, in addition to pregnenolone, Guarneri *et al.* (1994) detected DHEA, progesterone, deoxycorticosterone, THPROG, and 3 $\alpha$ , 5 $\alpha$ -tetrahydrodeoxycorticosterone. Treatment of primary rat brain extracts with L-ascorbate boosted apparent levels of allotetrahydrodeoxycorticosterone and DHEA in addition to those of pregnenolone (Roscetti *et al.*, 1998).

#### **BOX 15.4**

##### **3 $\alpha$ -DEHYDROGENATION AND 5 $\alpha$ -REDUCTION; 17 $\alpha$ AND 20 $\beta$ DEHYDROGENASE ACTIVITIES OF 3 $\alpha$ HSDS**

A second but distinct pathway, mediated by 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ HSD), often in conjunction with stereospecific reduction of the 4–5 unsaturated bond (adding a 5 $\alpha$  hydrogen), produces a separate class of steroids from the 3-oxo molecules produced by 3 $\beta$ -HSD. For the most part, such steroids are produced from progesterone, either directly to produce 5 $\alpha$ -3 $\alpha$ -hydroxy tetrahydroprogesterone (THPROG) or first via steroid 21 $\beta$ -hydroxylase (CYP21) to deoxycorticosterone, and then to 5 $\alpha$ , 3 $\alpha$ -hydroxytetrahydrodeoxycorticosterone (THDOC). Testosterone is also a substrate (Figure 15.1), producing the potent anesthetic steroid 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol.

Despite the functional linkage implied between the 5 $\alpha$  reduction and 3 $\alpha$ HSD steps, the enzymes are expressed separately. 5 $\alpha$  reductase activity is found in many brain regions, particularly the hypothalamus and pons/ medulla (Roselli and Snipes, 1984; Bertics *et al.*, 1987; Li *et al.*, 1997) and appears to be primarily neuronal (Melcangi *et al.*, 1993, 1994). There exist at least 2 distinct 5 $\alpha$  reductase genes. The lower affinity type I enzyme is widely expressed in adult brain, while the type II is expressed at significant levels during

development, particularly in zones of neuronal proliferation and differentiation but, in the adult, only low mRNA levels were detected (Lauber and Lichtensteiger, 1996).

In contrast, 3 $\alpha$ HSD activity is highest in olfactory bulb, with moderate levels in hypothalamus, cortex and cerebellum, and lower levels in limbic, thalamic, midbrain and brain stem structures (Cheng *et al.*, 1994). The enzyme is predominantly expressed in astrocytes (Melcangi *et al.*, 1993, 1994). However, four types of 3 $\alpha$ HSD enzyme that have been cloned in human (Matsuura *et al.*, 1998). Type I is absent from brain, types II and III are expressed (Channa *et al.*, 1995; Matsuura *et al.*, 1998) while expression of the type IV enzyme in brain has not been studied. However, the type II enzyme harbors dual 3 $\alpha$ - and 17 $\beta$ -HSD activities and only poorly converts dihydrotestosterone to 3 $\alpha$ -hydroxy androstenediol (Lin *et al.*, 1997), while the type III enzyme is more active on prostaglandins than it is on 3 $\alpha$ - or 17 $\beta$ -hydroxylated molecules (Matsuura *et al.*, 1998).

The difference in cellular expression and regional distribution could suggest that transport of intermediates is pivotal to the generation of "5 $\alpha$ -3 $\alpha$ " steroids in brain, or that these steroids make a significant contribution during development. For instance, maternal progesterone may suppress arousal of the fetal brain (Crossley *et al.*, 1997). In the adult it is unclear whether the "5 $\alpha$ -3 $\alpha$ " anesthetic steroids play any functional role outside pregnancy. Stuerenburg *et al.* (1997) were unable to detect conversion of pregnenolone to progesterone in cortex *in vitro* while Young *et al.* (1996) failed to detect THPROG in normal mouse brain, but reported nanomolar concentrations in male mice treated with trilostane, a 3 $\beta$ -HSD inhibitor, and consequent suppression of aggressive behavior. Curiously, administration of progesterone brought about an even more dramatic increase in brain THPROG, but had no antiaggressive effect. It may perhaps be suggested that THPROG activation of GABA<sub>A</sub> receptors is unlikely to play a major role in the adult brain; the same may not be true of 3 $\alpha$ -hydroxy-5 $\alpha$  molecules derived from adrenal steroids and further metabolized in the brain.

It is of note that many 3 $\alpha$ -HSD enzymes are relatively promiscuous in the site-specificity of the reaction catalyzed. Type II 3 $\alpha$ -HSDs also display 17 $\beta$ -HSD activity. Other enzymes classified as 3 $\alpha$ -HSDs are members of the short-chain dehydrogenase/reductase family, rather than being aldo-keto/oxo reductases, and also possess 20 $\beta$ -hydroxylation activity (Pocklington and Jeffrey, 1968; Sweets *et al.*, 1980; Ohno *et al.*, 1991; see also Penning *et al.*, 1997). 3 $\alpha$ -/20 $\beta$ -HSD enzyme is reported in brain (Kobayashi *et al.*, 1995) but no detailed studies have been reported. Finally, an epimerase catalyzing the isomerization of 3 $\alpha$ -steroids to 3 $\beta$ -steroids has been reported in rat brain and other tissues (Huang and Luu-The, 2000). In view of the markedly different properties of 3 $\alpha$ - versus 3 $\beta$ -steroids (for instance in GABA<sub>A</sub> modulation and anesthetic properties) this one enzyme could be a critical arousal determinant.

### Are brain steroid levels independent of peripheral synthesis?

A final pointer for local steroidogenesis in the rodent nervous system is that brain steroid levels can vary independently of their concentrations in blood. The key thread in the argument is that adrenalectomy (adx) plus gonadectomy (gdx) can have little effect on

steroid levels in brain. For instance, adx/gdx resulted in a 10-fold reduction in plasma steroid levels but only a slight depression (by 30–40%) in brain levels (Korneyev *et al.*, 1993). In the same study peripheral benzodiazepine receptor ligands were able to increase brain pregnenolone levels (but not those of DHEA), but were without effect on plasma pregnenolone levels. This suggests that at least some brain synthesis of pregnenolone takes place *in vivo*.

However, the interpretation of such studies is not always easy. Firstly, it is difficult to prove that surgical gdx/adx is complete, with latent residua potentially of adrenal tissue becoming hypertrophic to replace the removed organ. The operation is also not neutral to the animal. In one study, adx/gdx increased rather than reduced brain steroid levels and, like stress, roughly doubled brain pregnenolone and DHEA (Robel *et al.*, 1987). Secondly, studies on adx/gdx animals could be misleading if there are other non-brain sites of steroid synthesis, as noted by Le Goascogne *et al.* (1995). For instance, steroidogenesis has been detected in gut and skin during development (Keeney *et al.*, 1995) and key enzymes P450<sub>scc</sub>, CYP17 and CYP21 are expressed in normal human skin (Slominski *et al.*, 1996); some brain steroids in adx/gdx animals could potentially derive from such tissues. Finally, peripheral steroids tend to accumulate rapidly and selectively in fatty tissues such as brain (see Cheney *et al.*, 1995) and brain levels of, e.g., pregnenolone can exceed plasma levels by a factor of 10–50 (Robel *et al.*, 1987; Korneyev *et al.*, 1993; Cheney *et al.*, 1995).

With these caveats in mind the data for local steroid production in brain are not unambiguous. Cheney *et al.* (1995) focused on brain concentrations of progesterone (PROG) and its immediate derivatives 5 $\alpha$ -dihydroprogesterone (DHPROG) and allopregnanolone (tetra-hydroprogesterone). Though concentrations were markedly higher in brain than in plasma, adx/gdx produced parallel decreases in brain and plasma PROG, suggesting that the majority of brain PROG derives from peripheral tissues. In the same study, adx/gdx was not accompanied by parallel declines in brain and plasma levels of allopregnanolone and DHPROG. Unexpectedly, adx/gdx produced decreases of 30–50% (allopregnanolone) and 70% (DHPROG) in brain, but failed to reduce plasma levels. This could mean that these steroids only enter the brain from the periphery if produced in excess. If so, this observation would argue against significant *de novo* brain synthesis of allopregnanolone and PROG from cholesterol. This interpretation is indicated by the fact that intravenous pregnenolone sulfate increased brain pregnenolone levels massively (50-fold); allopregnanolone levels increased 4–5 fold but, significantly, the increase factor was greater in adx/gdx animals (7–8 fold) (Cheney *et al.*, 1995). These data suggest that, at most, only a modest proportion of brain allopregnanolone derives from local metabolism of progesterone.

Evidence that brain concentrations do not decline following adx+gdx (above) is crucial to the argument for brain steroid synthesis, but in one report not only did brain levels remain steady or increase, but blood levels of DHEA also increased following operation. Blood DHEA sulfate levels in the intact animal were 0.26ng/ml. This was unaffected by sham surgery (0.28ng/ml) but rose to 0.36ng/ml 15 days post adx+gdx (Corpechot *et al.*, 1981). It would be unwise, therefore, to rely on such data to support the case for steroidogenesis in brain.

On balance, if some *de novo* steroid synthesis does take place in rodent brain takes place, it is not very efficient. This is indicated by: (a) low levels of expression of key steroidogenic

enzymes; (b) marginal levels of pregnenolone and DHEA synthesis in brain; and (c) failure of pregnenolone and DHEA levels to be independent of blood levels. If this is true, one must suppose that the majority of brain steroids (but not sterols) in the adult derive from the circulation. Nonetheless, a key issue is whether some local synthesis does take place, because peripherally- and locally-derived steroids could have quite different metabolic fates. Some derivative steroids are extremely potent regulators (for instance molecules with the  $3\alpha$ ,  $5\alpha$  configuration can activate GABAA receptors at concentrations orders of magnitude lower than, e.g., the high concentrations of DHEA and pregnenolone). Low levels of steroid synthesis could have dramatic effects fast metabolism of steroids close to their sites of action could play a major role in modulating brain function (Balthazart and Ball, 2000). For *de novo* steroidogenesis in brain, while the interim verdict is “*not proven*”, the jury is still out.

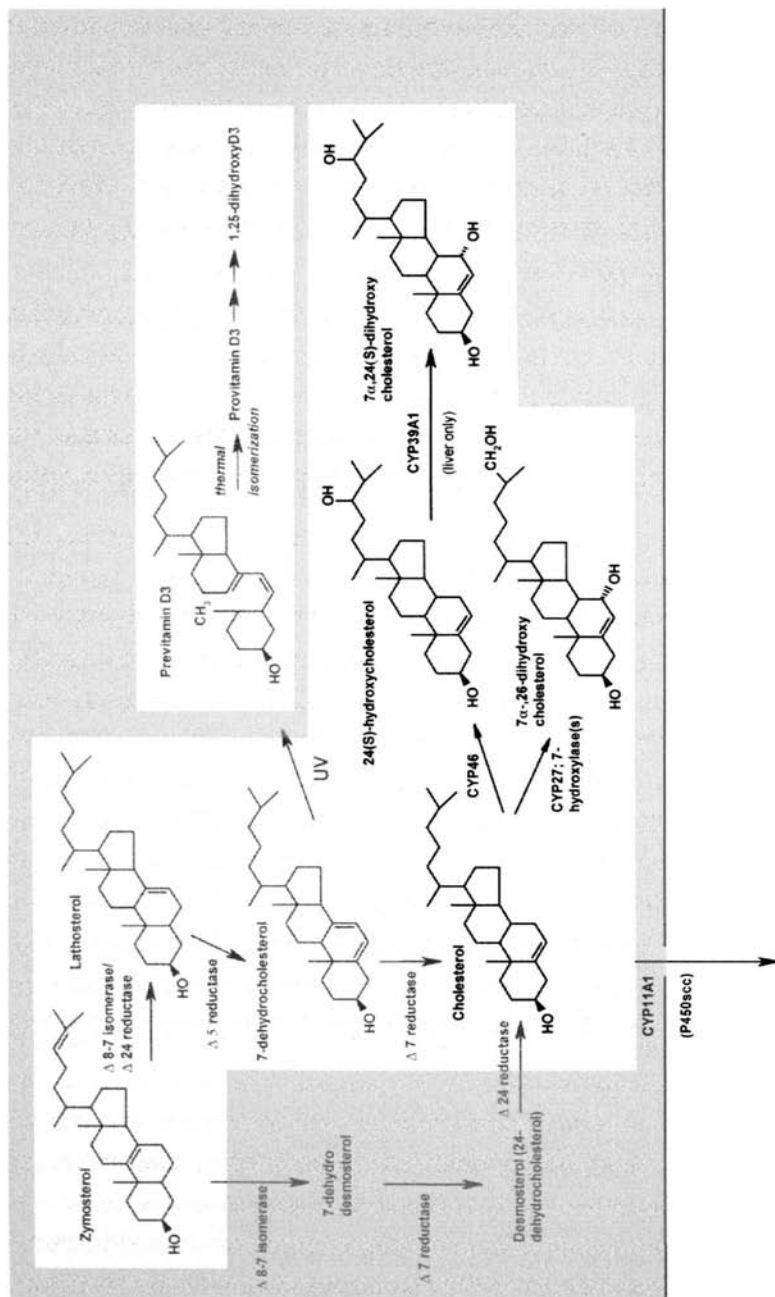
### LOCAL STEROID METABOLISM AND THE MITOCHONDRION

The key step in steroid synthesis is the removal of the side-chain of cholesterol to produce the first steroid molecule, pregnenolone (Figure 15.1). What is unusual is that the enzyme responsible, P450<sub>scc</sub> or CYP11A1, is located in mitochondria, and substrate delivery to this enzyme is a key regulatory step (discussed later). While the vast majority of P450s are targeted to microsomes and, though some unusual enzymes have dual targeting specificity (e.g., CYP1A1: Addya *et al.*, 1997; Bhagwat *et al.*, 1999; CYP2E1: Neve and Ingelman-Sundberg, 1999), only three major P450 types are predominantly localized to mitochondria. These are the cholesterol side-chain cleavage enzyme (CYP11A1), the 27-hydroxylase of cholesterol (CYP27; also 25-hydroxylase of Vitamin D), and two enzymes active at the 11-position including the corticosterone-synthesizing enzyme CYP11B1 (with additional 18-hydroxylation activity) and aldosterone synthase CYP11B2.

All these enzymes are expressed in brain, though at modest levels (see text and Box 15.5). Although co-expression in the same cells has not been formally demonstrated, it is possible that the improved solubility of hydroxycholesterols may facilitate delivery to P450<sub>scc</sub> (e.g., Jones and Hsueh, 1982; Brand *et al.*, 1998) and 27-hydroxylation could therefore cooperate with side-chain cleavage in steroid synthesis. Note that the cholesterol 24(S) hydroxylase, CYP46, is microsomal (Lund *et al.*, 1999).

Co-expression of P450<sub>scc</sub> (CYP11A1) and CYP11B (Box 15.5) would suggest preferential synthesis of 11 $\beta$ -hydroxypregnenolone, a conversion that has some antecedents (Maschler *et al.*, 1975; Murphy, 1981; see also Ainsworth and Nitchuk, 1975) but which is not generally regarded as a major route of steroid metabolism. However, 3 $\beta$ -hydroxysteroid dehydrogenase is distributed richly in both the mitochondrial and microsomal compartments (see Thomas *et al.*, 1999), arguing that a major local product could be 11 $\beta$ -hydroxyprogesterone (Figure 15.1), a natural glucocorticoid receptor ligand and potential substrate for further local metabolism.





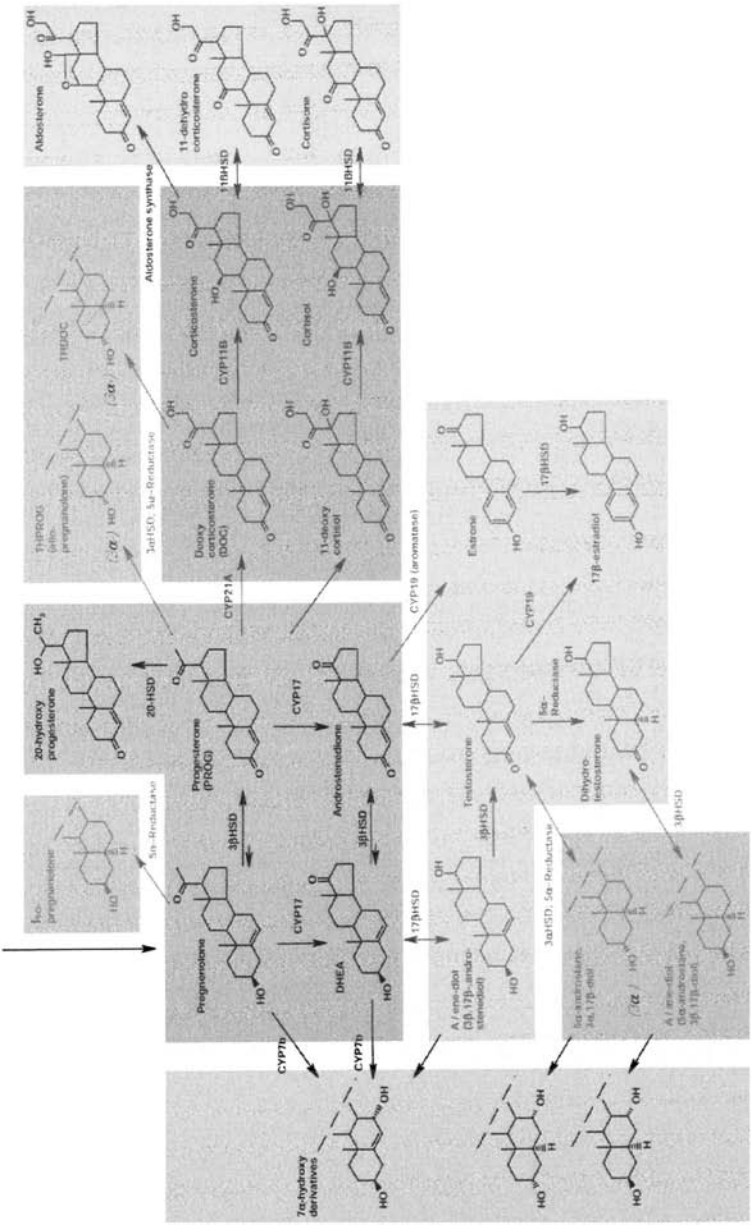


Figure 15.1 Pathways of steroid and sterol metabolism in brain (simplified)

**BOX 15.5****11 $\beta$ -HYDROXYLATION/ALDOSTERONE SYNTHASE, AND 11 $\beta$ -HSD**

Adrenal corticosteroids: glucocorticoids (cortisol in humans and most mammals, corticosterone in rats and mice) and mineralocorticoids (aldosterone), have potent actions on the central nervous system, affecting a wide range of electrophysiological, structural and metabolic parameters and thereby altering functions such as mood, cognition, neuronal development and survival (de Kloet, 1991).

**11 $\beta$ -hydroxylation and corticosteroid synthesis.** Until recently glucocorticoid synthesis via 11 $\beta$ -hydroxylation of deoxycorticosterone and deoxycortisol was thought to be the exclusive property of the adrenal. However, the 11 $\beta$ -hydroxylase (CYP11B1) is expressed in brain, as assessed by immunohistochemistry (Ozakie *et al.*, 1991) and RT-PCR (Gomez-Sanchez *et al.*, 1996; MacKenzie *et al.*, 2000), and confirmed by *in situ* hybridization, albeit at low levels (Erdmann *et al.*, 1995, 1996). Ozakie *et al.* (1991) reported enzyme activity in brain (cortical white matter) at about 15% of the level seen in adrenal, while Gomez-Sanchez *et al.* (1996) describe conversion of deoxycorticosterone to corticosterone in brain extracts. However, Stromstedt and Waterman (1995) suggest that CYP11B1 is expressed in rat brain but not in mouse brain.

Aldosterone is generally produced by concerted 11 $\beta$ -hydroxylation and 18 hydroxylation/dehydrogenation that converts inert deoxycorticosterone directly to aldosterone. The enzyme responsible, aldosterone synthase (P450Aldo; CYP11B2), appears also to be expressed in some brain regions, including hippocampus (Gomez-Sanchez *et al.*, 1997); these authors detected aldosterone synthesis in brain minces. Together these suggest that at least some glucocorticoid and mineralocorticoid synthesis might take place in brain, though the prevalent view is that glucocorticoids principally derive from the circulation.

**Glucocorticoid inactivation/reactivation by 11 $\beta$ -hydroxysteroid dehydrogenase.** Plasma glucocorticoid levels are tightly regulated by the hypothalamic-pituitary-adrenal (HPA) axis and its suprahypothalamic inhibitory regulators. Notable is the hippocampus which highly expresses both corticosteroid receptors, the lower-affinity glucocorticoid receptor (GR) and the higher affinity mineralocorticoid receptor (MR), and appears to act in glucocorticoid inhibitory control upon HPA activity (Jacobson and Sapolsky, 1991).

Until recently the main determinants of corticosteroid action within the brain were thought to be the prevailing levels of the steroids, mostly derived from the circulation, and the relevant densities of the two intracellular receptors, GR and MR. Recently it has become apparent that a hitherto arcane enzyme complex, 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD), plays an important role in determining intracellular glucocorticoid action (Seckl, 1997). 11 $\beta$ -HSD catalyzes the interconversion of active physiological 11-hydroxy glucocorticoids (cortisol, corticosterone) and their inert 11-oxo forms (cortisone, 11-dehydrocorticosterone). The latter are not known to bind to receptors. The physiological importance of the enzyme was only recognized a decade ago when renal 11 $\beta$ -HSD was found to inactivate glucocorticoids and prevent them from binding to the innately non-selective MR, allowing aldosterone alone to bind (Edwards *et al.*, 1988; Funder *et al.*, 1988). In the absence of the enzyme or the presence of liquorice-based inhibitors, cortisol gains access to and activates MR causing sodium retention, potassium loss and severe hypertension. Subsequently, it was discovered that there are

two distinct 11 $\beta$ -HSD isozymes, the product of distantly related genes, which play very different roles.

11 $\beta$ -HSD Type 2 is a high-affinity, NAD-dependent 11 $\beta$ -dehydrogenase, which rapidly inactivates glucocorticoids to their inert 11-oxo forms. It is this activity that engenders MR specificity in the kidney (Albiston *et al.*, 1994). Mutations in the 11 $\beta$ -HSD2 gene result in the rare hypertensive syndrome of apparent mineralocorticoid excess (Mune *et al.*, 1995) and transgenic mice homozygous for targeted disruption of the 11 $\beta$ -HSD2 gene show an analogous hypertensive phenotype due to illicit occupation of MR by corticosterone (Kotelevtsev *et al.*, 1999). 11 $\beta$ -HSD2 is also highly and widely expressed in the developing mid-gestational brain at a time preceding that of MR expression, but coinciding with GR expression (Brown *et al.*, 1996; Diaz *et al.*, 1998). It has thus been hypothesized that this may be important in attenuating glucocorticoid effects on neuronal and glial development. Postnatally, 11 $\beta$ -HSD2 is only expressed in a few selective regions of the rat brain, such as the nucleus tractus solitarius, the subcommissural organ and scattered neurons in the central nucleus of the amygdala (Robson *et al.*, 1998). In these regions aldosterone appears to exert selective effects on salt appetite and blood pressure which are not mimicked by corticosterone (Gomez-Sanchez, 1986; McEwen *et al.*, 1986), suggesting that 11 $\beta$ -HSD2 protected MR exists in these sites. This notion, supported by the hypertensive effects of central infusion of enzyme inhibitors (Gomez-Sanchez and Gomez-Sanchez, 1992), nevertheless remains to be formally established.

In contrast, 11 $\beta$ -HSD Type 1 is widely expressed in the adult CNS including the hippocampus, and predominantly in neurons, as well as in the pituitary and hypothalamus (Moisan *et al.*, 1990, 1992; Lakshmi *et al.*, 1991). Whereas the enzyme is bidirectional in cell homogenates, in intact cells it is often a predominant 11 $\beta$ -reductase (Jamieson *et al.*, 1995) (although other studies have argued that the hippocampal enzyme catalyzes bidirectional interconversion; Jellink *et al.*, 1999). This reaction direction, far from inactivating glucocorticoids, regenerates active steroids from inert circulating 11-oxo forms. Recent data suggest that cortisone and 11-dehydrocorticosterone levels are around 10–50nmol/l in human and rat plasma. The 11-oxo steroids circulate largely unbound to plasma proteins, unlike the active 11-hydroxy glucocorticoids, which are around 95% bound to CBG and albumin (Dunn *et al.*, 1981). Thus, although total cortisol or corticosterone levels are between 20 and perhaps 500nmol/l, with marked diurnal variation, “free” levels are lower than those of 11-oxo steroids for at least part of the day. The potential importance of 11 $\beta$ -HSD1 in the CNS remained moot until studies in primary hippocampal neurons showed predominant 11 $\beta$ -reductasemediated glucocorticoid reactivation (Rajan *et al.*, 1996), so contributing to glucocorticoid neurotoxicity (Ajilore and Sapolsky, 1999). The recently described 11 $\beta$ -HSD1 knock-out mouse (Kotelevtsev *et al.*, 1997) should allow determination of any biological importance of this *in vivo*. Indeed, the mutant mice show elevated basal corticosterone levels, suggesting that glucocorticoid negative feedback control of the HPA axis is attenuated, raising the possibility that 11 $\beta$ -HSD1 could contribute to age-related changes in HPA axis activity (Meaney *et al.*, 1993). Indeed, very recent data directly support the notion of blunted glucocorticoid feedback control of the HPA axis in 11 $\beta$ -HSD1 null mice (Harris *et al.*, 2000). In this light it is intriguing that hippocampal 11 $\beta$ -HSD1 activity is downregulated by chronic stress (Jamieson *et al.*, 1997), perhaps acting to help to protect vulnerable neurons from the deleterious effects of chronic glucocorticoid excess. 11 $\beta$ -

reductase represents a potential target for manipulating glucocorticoid action therapeutically.

### STEROID HORMONE NUCLEAR RECEPTORS ARE ABUNDANT IN BRAIN

Peripherally-produced molecules that can access the brain include the sex steroids (testosterone, DHT, estradiol, progesterone), adrenal steroids including the corticosteroids (glucocorticoids and aldosterone), pregnenolone and dehydroepiandrosterone (DHEA), and non-steroidal molecules including the hydroxycholesterols, vitamin D, thyroid hormones (triiodothyronine (T3) and thyroxine flio (T4)). These are thought to bind to classic steroid hormone receptors present in brain cells, whereby they modulate transcription. The family of steroid hormone receptors includes the androgen receptor (AR), estrogen receptors (ER $\alpha$  and ER $\beta$ ), the receptors for glucocorticoids and mineralocorticoids (GR and MR), progesterone (PR), vitamin D (VDR), thyroid hormone (TR subtypes) and other diverse members including similar but poorly-characterized receptors such as the estrogen-receptor related molecules ERR1 and ERR2, and also receptors responding to oxysterols (e.g., LXR $\alpha$ , FXR, CAR- $\beta$ ), fatty acids and prostenoids (PPARs) (reviewed by Kumar and Thompson, 1999; Shibata *et al.*, 1997; see in particular the evolutionary trees assembled by Laudet, 1997; Laudet *et al.*, 1992; Baker, 1997). Many if not all of these receptors are expressed in the brain, with expression of AR, ER $\alpha$ , ER $\beta$ , GR and MR being recorded in hypothalamus, cortical and limbic regions, including the hippocampus, and other brain structures.

According to the traditional view of steroid hormone action, ligand binding to the cognate cytoplasmic receptor is followed by transport of the complex to the nucleus wherein transcription of specific hormone-responsive genes is induced or repressed. This is exemplified by the glucocorticoid hormones.

Two receptors exist, GR and MR, with different affinities for the major circulating glucocorticoid, corticosterone in rodents and cortisol (hydrocortisone) in man. MR binds corticosteroids avidly, while the lower affinity receptor GR only becomes fully occupied under conditions of stress. The principal effect of MR or GR ligand binding is stimulation or repression of specific gene expression (reviewed by Vreugdenhil *et al.*, 1996; de Kloet *et al.*, 1998); note that the precise classes of genes induced by MR and GR are overlapping but are to some extent distinct. In addition to genomic actions on transcription, many steroids have fast actions on neuronal excitability that cannot be explained by relatively slow changes in transcription. For other steroids (e.g., pregnenolone and DHEA) no nuclear receptors are known; instead these appear to influence neuronal activity by modulating non-nuclear receptors. These aspects are considered separately.

## MEMBRANE-ASSOCIATED STEROID RECEPTORS

While the molecular evidence argues that steroids target specific cytosolic receptors that subsequently translocate to the nucleus, the pharmacologic evidence is sometimes very different. Extremely rapid effects on neuronal excitability (seconds to minutes) can be detected, a timescale inconsistent with *de novo* transcription of hormone-responsive genes. To give one example, estradiol induces rapid changes in the electrical activity of cells without evident functional nuclear estrogen receptors (Gu *et al.*, 1999). Other steroidal molecules have been shown to influence neuronal function despite coupling to large molecules such as serum albumin, arguing that, in general, some functional receptors for steroid hormones reside at the cell surface (reviewed by Schmidt *et al.*, 2000). This underlines an earlier report of a specific high affinity cell-surface receptor for aldosterone (Wehling *et al.*, 1993) and a cell-surface target for corticosterone in amphibian brain (Moore and Orchinik, 1994). Wagner *et al.* (1999), working with novel inhibitors of the progesterone and glucocorticoid receptors RTI3021–012 and 022, found that, while these two new molecules displayed avid binding to GR, they failed to block corticosteroid action, possibly suggesting that some glucocorticoids may also act through non-GR pathways so far uncharacterized. Gametchu *et al.* (1999) discuss a cell-surface variant of the glucocorticoid receptor GR that they term mGR; this species appears to be an isoform of the classic nuclear receptor. It is of note that glucocorticoid entry to cells appears to rely on a specific and saturable uptake system at the cell surface (Allera and Wildt, 1992a, b; Lackner *et al.*, 1998), but photoaffinity labeling revealed a 53kD membrane protein in rat liver plasma membrane (Ibarrola *et al.*, 1992) that was competed by cortisol, deoxycorticosterone and progesterone. This is probably distinct from mGR on molecular weight grounds.

Evidence has also been provided for a cell surface estradiol receptor. Monje and Boland (1999) report that estradiol binding to the high-affinity surface receptor of rabbit uterus was reduced by anti-ER antibody; this would suggest that the cell-surface receptor comprises a structurally-modified form of the classic nuclear receptor ER $\alpha$ . However, this study does not rule out the possibility of a discrete class of cell surface estrogen receptors unrelated to the nuclear receptors ER $\alpha$  and ER $\beta$ . An intriguing report by Razandi *et al.* (1999) describes how 2% of ER $\alpha$  or ER $\beta$  appears at the cell surface, and moreover is functionally coupled to inositol polyphosphate production and adenylyl cyclase activity. Clarke *et al.* (2000) report that ER $\alpha$  immunoreactivity is principally associated with neurites (and not the nucleus) of rat hippocampal neurons, arguing for non-genomic actions at the cell membrane. Molecular characterization of the inferred cell surface glucocorticoid and estrogen receptors remains an important research objective and if, as so far seems likely, they derive from the classic nuclear receptors, the elucidation of their cell-surface signaling pathways may provide some surprises.

## NEUROSTEROID ACTION AT NEUROTRANSMITTER RECEPTORS

Some steroids, such as DHEA and pregnenolone, seem to lack a nuclear receptor, though the plethora of "orphan" nuclear receptors affords many candidates. There have been reports of high-affinity DHEA binding sites in peripheral tissues such as T-cells (Meikle *et al.*, 1992; Okabe *et al.*, 1995), cervical fibroblasts (Imai *et al.*, 1992) and liver cytosol (Yamada *et al.*, 1994). In the absence of demonstrated functionality these might possibly represent transport proteins rather than receptors per se (although it may be incorrect to make a firm distinction between the two). However, it has been well established that DHEA and structurally-related steroids can modulate neuronal function via an interaction with cell membrane receptors and ion channels.

Diverse neurotransmitter receptors have been shown to respond to steroids (including the AMPA, glycine, kainate, nicotinic acetylcholine, NMDA and oxytocin receptors; reviewed by Rupprecht and Holsboer, 1999). However the best studied interaction is with the ligand-gated ion channel responding to the principal inhibitory neurotransmitter, gamma-amino butyric acid (GABA).

Two types of steroid-GABA receptor interaction have been described, agonistic and antagonistic, dependent on the structure of the steroid ligand. Potentiation of the GABA<sub>A</sub> receptor by the synthetic steroid anesthetic alfaxalone was reported by Harrison and Simmonds (1984), while natural steroid metabolites of corticosteroids and progesterone that also bear a 3 $\alpha$ -hydroxy group are also potent agonists of the GABA<sub>A</sub> receptor (Majewska *et al.*, 1986). These latter steroids, produced by 3 $\alpha$ -reduction of deoxycorticosterone and progesterone, increase the inhibitory action of the GABA receptor, so suppressing neuronal activity and producing anesthetic, anticonvulsant, sedative and anxiolytic properties. Conversely, 3 $\beta$ -hydroxy steroids such as DHEA and pregnenolone are natural antagonists of the GABA<sub>A</sub> receptor (Majewska *et al.*, 1990), thus promoting neuronal activity (reviewed by Majewska *et al.*, 1992; Lambert *et al.*, 1995, 1996; Belelli *et al.*, 1999). Metabolic switching between 3 $\beta$ -hydroxylated steroids that antagonize the GABA<sub>A</sub> receptor (such as pregnenolone and DHEA) and 3 $\alpha$ -hydroxylated steroids (particularly those that are also 5 $\alpha$  reduced) may represent an additional level of control of neuronal activation in the brain *in vivo*.

As discussed elsewhere, it is noteworthy that the binding site on the GABA<sub>A</sub> receptor complex that interacts with steroids appears to be distinct from the modulatory binding sites on this same receptor for either benzodiazepines or barbiturates. Recent data also suggest that the binding sites for steroid agonists and antagonists may differ according to the subunit composition of the GABA<sub>A</sub> receptor (Maitra and Reynolds, 1998).

Steroid modulation of the N-methyl D-aspartate (NMDA)-type channel for the excitatory amino acid glutamate has also been recorded. In particular, pregnenolone appears to enhance the action of the NMDA receptor (Wu *et al.*, 1991), while an anesthetic 3 $\alpha$ -hydroxysteroid is reported to antagonize the same receptor (Park-Chung *et al.*, 1997). Thus, natural 3 $\beta$ -hydroxysteroids such as pregnenolone enhance neuronal excitability in two ways, by dampening inhibitory GABA<sub>A</sub> channel activity and by boosting excitatory NMDA

currents. Anesthetic  $3\alpha$ -hydroxysteroids have the reverse action, enhancing GABA<sub>A</sub> channels and inhibiting NMDA activity, suggesting that the local ratio of  $3\alpha$ - and  $3\beta$ -hydroxysteroids is critical to neuronal function. As noted earlier, steroid modulation of neurotransmitter receptors is found on many ligand-gated ion channels and is not restricted to the GABA and NMDA receptors (reviewed by Rupprecht and Holsboer, 1999).

A further level of complexity is added by the finding that neurosteroids may also act via intracellular targets. Two such targets have come to the fore: the peripheral benzodiazepine receptor and the sigma site. Both seem to be involved, perhaps counter-intuitively, in aspects of sterol transport and metabolism, and have the potential to be modulated by both sterols and steroids. These two targets are important because ligand binding may, by circuits that are largely unknown, result in alterations to ion channel activity.

### THE PERIPHERAL BENZODIAZEPINE RECEPTOR; CHOLESTEROL TRANSPORT

Benzodiazepines, exemplified by diazepam, are potent anticonvulsant and anxiolytic agents and are widely used in the clinic. These selectively target and generally enhance the activity of GABA<sub>A</sub> receptors at the cell surface, though regional differences in subunit affinity and expression may explain somewhat diverse pharmacologic actions (reviewed by Teuber *et al.*, 1999; Whiting *et al.*, 1999). However, biochemical studies revealed selective benzodiazepine binding in tissues not known to express functional GABA receptors, giving rise to the concept of the “peripheral benzodiazepine receptor”, PBR (also referred to as the “omega” receptor).

The cloned PBR (Sprengel *et al.*, 1989) polypeptide is an 18 kD protein with 5 trans-membrane domains stationed on the outer mitochondrial membrane and widely expressed in steroidogenic tissues, liver and brain. The receptor turns out to be a cholesterol transport protein that can cooperate with StAR-type sterol transporters (Sugawara *et al.*, 1997), probably including brain MLN64, to translocate cholesterol from the outer to the inner mitochondrial membrane (Besman *et al.*, 1989; Krueger and Papadopoulos, 1990). Thus, PBR delivers cholesterol to the key initial enzyme in steroid synthesis, mitochondrial P450<sub>scc</sub> (CYP11A1), and also the mitochondrial cholesterol 27-hydroxylase, CYP27. Cholesterol, a highly hydrophobic molecule, might be expected to diffuse freely in membranes, but this is not the case. In fact, membranes can act as an impermeable cholesterol barrier; this is best illustrated by the near-total block of steroid synthesis in individuals harboring mutations in the intracellular cholesterol transport protein StAR (see [Chapter 5](#)). Indeed, all aspects of cholesterol movement seem to require dedicated transport systems (reviewed by Liscum and Munn, 1999). Thus PBR ligands can dramatically increase the rate of steroid synthesis in diverse tissues (Papadopoulos and Brown, 1995) and also in brain (McCauley *et al.*, 1995).

Although PBR is thought to be regulated by porphyrins (hemin and protoporphyrin IX; Verma *et al.*, 1987), an insight into the role of PBR was provided by the characterization of an endogenous polypeptide ligand for the receptor. This is a conserved 87/107 amino acid polypeptide that competes with diazepam for binding, hence its name: “diazepam binding



inhibitor" (DBI) or endozapine (Gray *et al.*, 1986; Webb *et al.*, 1987; Costa and Guidotti, 1991). On binding PBR, the DBI polypeptide (like benzodiazepines themselves) stimulates cholesterol transport and promotes steroidogenesis (e.g., Besman *et al.*, 1989; reviewed by Papadopoulos *et al.*, 1997); disruption of the PBI gene in cultured cells prevents cholesterol transport and steroid synthesis (Culty *et al.*, 1999). Although DBI appears to lack a secretion signal sequence, there are alternative forms, some of which are membrane-associated (Webb *et al.*, 1987; Todaro *et al.*, 1991). DBI is widely expressed during development (Burgi *et al.*, 1999), in the brain and peripheral tissues (Alho *et al.*, 1988), and binds to both GABA<sub>A</sub> and PBR targets (reviewed by Barbaccia *et al.*, 1990; Costa and Guidotti, 1991; Papadopoulos *et al.*, 1991). DBI is predominantly non-neuronal in origin (Tong *et al.*, 1991); potentially, DBI released from glia could act at GABA<sub>A</sub> receptors to modulate neuronal metabolic activity. Such a *trans* action has not been demonstrated.

Drug targeting at PBR could be dismissed as purely circumstantial, except that binding is reported to elicit similar anticonvulsant and anxiolytic effects to binding at GABA<sub>A</sub> (Sanger *et al.*, 1994), and can similarly impair learning in rodents (McNamara and Skelton, 1991, 1992). In decided contrast, Gunther *et al.* (1995) report that mice lacking the gamma2 subunit gene of the GABA<sub>A</sub> receptor are refractory to diazepam. This would seem to argue that GABA<sub>A</sub>-gamma2, rather than PBR, provides the principal *in vivo* target for benzodiazepines. This conclusion should not go unchallenged because, while diazepam targets both the peripheral (omega/PBR) and central (GABA<sub>A</sub>) receptors, other agents with undoubted pharmacologic activity can selectively target the peripheral receptor (e.g., Ro5-4864 and PK11195) or GABAA (e.g., clonazepam). Selective PBR ligands can exert potent amnesic, anxiolytic and convulsant effects *in vivo* (McNamara and Skelton, 1992; Romeo *et al.*, 1994; Ferrarese *et al.*, 1995; Okuyama *et al.*, 1999).

To complicate the picture, pharmacologic evidence argues for a multiplicity of omega/PBR-type binding sites in brain (e.g., Woods and Williams, 1996; Rao and Butterworth, 1997). The molecular basis of this diversity is not known. Interactions between PBR and accessory proteins such as PRAX-1 that are selectively expressed in some sub-regions of the brain (Galiegue *et al.*, 1999) could be responsible, as could different phosphorylation states of PBR (Whalin *et al.*, 1994).

Overall, this work raises a pressing question. By what route do alterations in sterol transport modify brain function? Sterol transport is likely to regulate local neurosteroid synthesis; benzodiazepine binding to PBR improves cholesterol delivery to P450<sub>sc</sub>, and these could act by boosting levels of GABA-active neurosteroids derived from pregnenolone, though the contribution of *de novo* steroid synthesis remains to be confirmed. Sterol transport may be intimately coupled with aspects of mitochondrial function. PBR copurifies with a family of related voltage-dependent anion channels (porins) of the outer mitochondrial membrane (McEnery *et al.*, 1992, 1993). It is not known whether PBR regulates these ion channels but we should note that DBI/endozepine seems to have two targets. Firstly, it modulates a cell-surface ion channel that harbors a steroid binding site. Secondly, it activates a mitochondrial transport system involved in steroid synthesis; plausibly it could also regulate mitochondrial membrane polarisation. Cross-talk between the two systems is likely (see below). GABA<sub>A</sub> agonists, like PBR ligands, were reported to increase pregnenolone synthesis in isolated rat retina (Guarneri *et al.*, 1995).

## SIGMA RECEPTORS, EMOPAMIL BINDING PROTEIN AND STEROL METABOLISM

The involvement of PBR in sterol transport, and its targeting by neuro-active drugs, argues that sterols and sterol/steroid metabolism may regulate brain function. This conclusion is reinforced and emphasized by the identification of further intracellular sites sensitive to steroidal molecules, the "sigma" receptor and the emopamil-binding protein (EBP).

Pharmacologic sigma ligands encompass a group of chemically unrelated neuroleptic and neuroprotective drugs, such as haloperidol, pentazocine and emopamil, used for example in treating schizophrenia (reviewed by Walker *et al.*, 1990). Although some such agents also target the dopamine receptor (e.g., haloperidol, trifluoperazine) or the NMDA receptor (e.g., ifenprodil), others seem to be fairly selective for the sigma site (e.g., [+] pentazocine, NE100, SR31747A, PD144418) though interaction with other receptors is not excluded. Interactions with (+)-opiates and phencyclidine have also been suggested.

Sigma sites have been pharmacologically classified into sigma-1 and sigma-2 sites (Hellewell and Bowen, 1990; Quirion *et al.*, 1992), with different distributions in brain (McCann *et al.*, 1994; Bouchard and Quirion, 1997). A sigma-3 receptor has also been suggested (Myers *et al.*, 1994) but the molecular basis for this diversity is not yet clear. In fact, recent cloning experiments have led to the discovery of two coding sequences which share at least some pharmacologic properties with the sigma-1 site. Although both have properties that strongly suggest an involvement in sterol metabolism, they are, perhaps surprisingly, unrelated to each other.

### The sigma-1 receptor

Clones encoding a *de facto* sigma-1 receptor were identified on the basis of affinity for sigma ligands including haloperidol and SR31747A (Hanner *et al.*, 1996; Kekuda *et al.*, 1996; Jbilo *et al.*, 1997). This 25kD protein has a perinuclear localization, harbors a single transmembrane domain, and shares very significant homology (~30% sequence identity) with the yeast Erg2 (ergosterol synthesis; C8-C7 sterol isomerase) enzyme. Puzzlingly, it has not yet been possible to demonstrate any catalytic activity associated with the cloned polypeptide (Jbilo *et al.*, 1997). No inherited deficits in sigma-1 have been reported.

### Emopamil-binding protein

This polypeptide was cloned on the basis of affinity for emopamil, a neuroprotective agent effective in cerebral ischemia. The emopamil binding protein (EBP) (Hanner *et al.*, 1995; Silve *et al.*, 1996), a 26kD protein containing four putative Tm segments and probably resident in the endoplasmic reticulum, has been demonstrated to encode a functional sterol C8-C7 isomerase that catalyzes the penultimate step in the synthesis of cholesterol (Silve *et*

*al.*, 1996a, b; Hanner *et al.*, 1996; Moebius *et al.*, 1996). Expression of human EBP in mutant yeast permitted functional reconstitution of cholesterol synthesis while rendering the yeast susceptible to growth inhibition by trifluoperazine (Silve *et al.*, 1996b). In addition to inhibition by 7-ketocholestanol (Paul *et al.*, 1998), the protein displays surprisingly high affinity for the non-steroidal anti-estrogen tamoxifen (Moebius *et al.*, 1997; Paul *et al.*, 1998). Deficiency in EBP/sterol isomerase has been associated with lipid abnormalities and skin and limb defects (CHILD syndrome; Grange *et al.*, 2000) and with X-linked chondrodysplasia (Conradi-Hunermann syndrome) (Derry *et al.*, 1999; Braverman *et al.*, 1999).

The relationship between sigma and EBP remains unclear (Moebius *et al.*, 1997). Although dissimilar in primary sequence, both bind some "sigma" ligands such as tridemorph with high affinity, and whereas haloperidol targets the mammalian sigma-1 receptor and not EBP, haloperidol inhibits cholesterol synthesis in yeast by targeting the endogenous EBP/sterol isomerase (Moebius *et al.*, 1996). The absence of apparent enzymatic activity associated with the sigma-1 polypeptide in yeast may be for technical reasons. As discussed (Moebius *et al.*, 1997), sigma-1 could be a sterol-binding regulatory protein or catalyze a novel conversion. At least some pharmacologic sigma ligands (e.g., SR31747) inhibit cholesterol synthesis and stop cell proliferation; the block can be overcome by exogenous cholesterol (Labit-Le Bouteiller *et al.*, 1998).

### **Are sterol (and steroid) targets generic sigma sites?**

The endogenous ligand of the pharmacologic binding site on sigma-1 is not known. Although neuropeptide Y has been discussed (reviewed by Wan and Lau, 1995), other evidence has argued that the endogenous ligand could be steroidal in nature (Su *et al.*, 1998). Corticosterone, deoxycorticosterone, testosterone and progesterone are reported to competitively inhibit the binding of pharmacologic sigma agents (Su *et al.*, 1988, 1990), and manipulation of systemic steroids affects behavioral changes mediated through the sigma-1 receptor (Phan *et al.*, 1999). There is every likelihood that neurosteroids can target the sigma-1 receptor (and probably EBP) in addition to their well-documented actions on cell surface ion channels. For instance (and as discussed further below), DHEA (and pentazocine) appears to improve memory function in mice by acting as a sigma agonist, while progesterone (and haloperidol) appears to be an antagonist and reduces cognitive performance (Maurice *et al.*, 1998). Similar conclusions were reached for DHEA by Bergeron *et al.* (1996) and Urani *et al.* (1998); in both cases the effects of DHEA were reversed by sigma antagonists (see also Monnet *et al.*, 1995). These studies must be interpreted with caution in view of possible pharmacologic overlap between sigma sites (sigma-1,-2) and EBP.

Extending the discussion, Jbilo *et al.* (1997) put forward the intriguing suggestion that sterol binding sites may constitute targets for sigma ligands. This would argue that other enzymes metabolizing, transporting or responding to sterols might furnish novel "sigma" sites. Among candidate polypeptides one might consider the four sterol sensing proteins discussed by Lange and Steck (1998) (Ptc, signal transduction; HMGCoA reductase,

cholesterol synthesis; NPC1, Niemann-Pick disease type 1 and sterol transport; and the SREBP-cleavage protein, SCAP). Also, the cholesterol synthesis enzymes listed by Fitzky *et al.* (1999), diverse sterol-metabolizing enzymes (including P450s and dehydrogenases), and diverse nuclear receptors (see below) including CPF (Nitta *et al.*, 1999); potential brain targets for steroids and sterols are summarized in Table 15.2. Transmembrane domains of cell-surface receptors interact with cholesterol and could afford further sigma sites. No pharmacologic evidence for sigma ligand targeting at such sites has yet been presented, but this situation seems ripe for change.

### STEROL SYNTHESIS AND METABOLISM IS CRUCIAL TO BRAIN FUNCTION

Targets for anxiolytics and anticonvulsants (e.g., benzodiazepines) and neuroleptics (e.g., haloperidol) include enzymes and transporters involved in sterol

**Table 15.2** Brain targets for steroids.

<i>Cell surface receptors</i>	<i>Sterol synthesis* enzymes and regulation</i>	<i>Transporters</i>	<i>Nuclear receptors</i>	<i>Signaling molecules</i>
GABA <sub>A</sub>	HMGCR	Peripheral benzodiazapine receptor (PBR)	GR, MR	<i>Sonic</i>
NMDA	HMGCS	NPC-1, -2	PR, AR	<i>Hedgehog</i> (Shh)
AMPA	Sterol 8-7 isomerase (sigma)	StAR, MLN64	ER $\alpha$ , $\beta$	<i>Patched</i> (Ptc)
Kainate	EBP	Caveolin	VDR, TR subtypes	
Glycine		FKBP56	ERR1,2, PPAR, PXR	
Acetyl choline			CAR, PXR, LXR, FXR	
sigma-2, sigma-3				
Oxytocin	SCAP/SREBP	Cyclophorins	SF-1, SXR, CPF, FTZ-F1	
mGR?		LDL-receptor family	NUR1, NUR77, COUP-TF	
		Apolipoproteins	LHR-1	
		B,E, other		
		<i>Tout-velu</i>		
		OPP1-6		

\* Other potential targets include enzymes involved in sterol and steroid metabolism, including cholesterol and steroid hydroxylases (CYPs 7, 11), 5 $\alpha$ -reductases and hydroxysteroid dehydrogenases (3 $\beta$ -HSD, 11 $\beta$ -HSD). HMGCR/S; hydroxy-methyl glutaryl CoA reductase/synthase.

transport and metabolism. This would suggest that sterol metabolism can play a regulatory role. This is confirmed by other genetic and pharmacologic evidence.

Different alleles at the locus encoding cholesterol delivery protein ApoE can in part determine the onset of Alzheimer's disease (Strittmatter *et al.*, 1993; Corder *et al.*, 1993; Saunders *et al.*, 1993), although the linkage is by no means perfect (Lannfelt *et al.*, 1994).

ApoE deficient mice are cognitively impaired (Gordon *et al.*, 1995) while transgenic mice overexpressing ApoE4 show age-dependent learning and memory deficits, but only significantly in females (Raber *et al.*, 1998). Cholesterol uptake receptor genes are also involved; loci encoding low-density lipoprotein (LDL) receptor related protein, LRP, or the very low-density lipoprotein (VLDL) receptor, have also been linked with late-onset Alzheimer's (Kang *et al.*, 1997; Okuizumi *et al.*, 1995; but see Okulzumi *et al.*, 1996). LRP-deficient mice are cognitively impaired (Van Uden *et al.*, 1999). Mutations affecting *de novo* cholesterol synthesis also have dramatic effects on cognition. Deficiency of 7-dehydrocholesterol reductase causes Smith-Lemli-Opitz syndrome (SLOS), characterized by developmental abnormalities, adrenal insufficiency and mental retardation (Waterham *et al.*, 1998; Wassif *et al.*, 1998; Moebius *et al.*, 1998). Inhibition of 7-dehydrocholesterol synthesis in weanling rats impairs later learning abilities (Xu *et al.*, 1998) while cholesterol synthesis inhibition during gestation produces developmental deficits reminiscent of SLOS (Lanoue *et al.*, 1997). Niemann-Pick disease type C ("NPC"), a condition associated with marked neurological deterioration (reviewed by Vanier and Suzuki, 1998), affects intracellular sterol transport mediated by the NPC1 gene product (Carstea *et al.*, 1991). Finally, deficiency of cholesterol 27-hydroxylation (CYP27) causes cerebrotendinous xanthomatosis, a disease characterized by mental retardation and atherosclerosis (Cali *et al.*, 1991; see also Leitersdorf *et al.*, 1993; Björkhem, 1994). However a similar deficiency in mice seems to have rather less pronounced consequences (Rosen *et al.*, 1998).

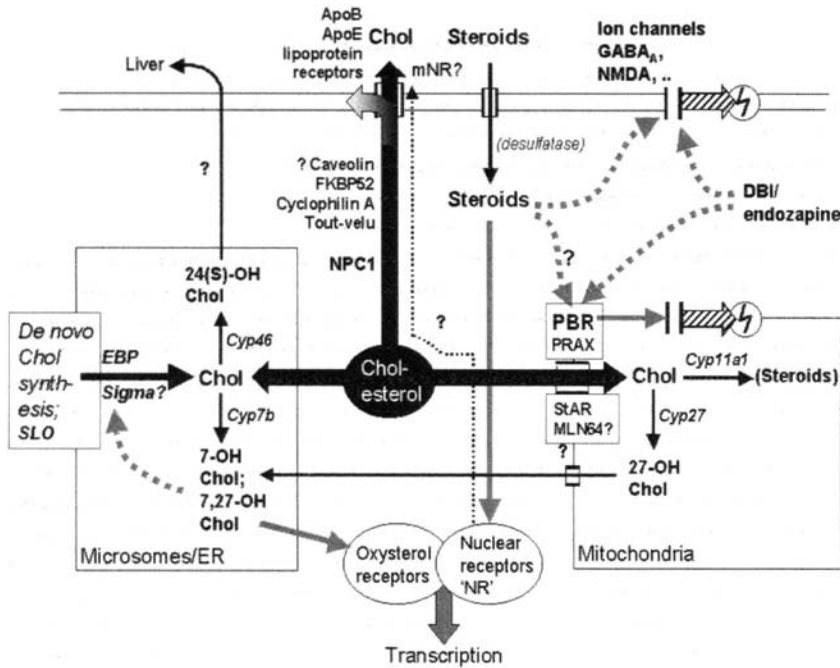
Some evidence argues that the potent neuroprotective drug FK506 (Dawson *et al.*, 1993; Sharkey and Butcher, 1994) may operate in cholesterol trafficking, in addition to its well-known action on calcineurin. Transport of cholesterol "rafts" and/or caveolae, cholesterol-rich domains in the cell membrane, is mediated by the FK506 binding protein FKBP52 (Gold *et al.*, 1999; also known as HSP56) in a complex with cholesterol, the cholesterol binding protein caveolin, and cyclophilins A and 40 (Uittenbogaard *et al.*, 1998). The immunosuppressive drug cyclosporin, that also targets cyclophilins, also seems to modulate cholesterol delivery to mitochondria (Dahlback-Sjöberg *et al.*, 1993) and has neurotoxic effects on chronic administration (Hauben, 1996; Gijtenbeek *et al.*, 1999). Significantly, the sigma receptor may bind tightly to cyclophilin A, as demonstrated by copurification of a cyclophilin fragment with sigma (Schuster *et al.*, 1994).

Cholesterol levels change in Alzheimer's disease and during FK506 treatment; blood cholesterol may in turn influence behavior (Fawcett *et al.*, 1997; Kaplan *et al.*, 1997). Circumstantially, cholesterol-derived vitamin D may subvert brain function (Keddie, 1987; Stumpf, 1988; Holick, 1995; Lansdowne and Provost, 1998); note that 25-hydroxylation of vitamins D is performed by the mitochondrial sterol 27-hydroxylase, CYP27 (Usui *et al.*, 1990; Guo *et al.*, 1993).

## OXYSTEROL AND STEROID SIGNALING INTERACTIONS

## Oxysterol metabolism in brain

It has been suggested that only low levels of *de novo* steroid synthesis occur in brain, but agents modifying sterol synthesis and metabolism can have profound effects on brain function. This underlines the importance of sterol metabolism in brain.



**Figure 15.2 Pathways of sterol and steroid signaling in brain.** CHOL, cholesterol; DBI, diazepam binding inhibitor; EBP, emopamil binding protein; ER, endoplasmic reticulum; PBR, peripheral benzodiazepine receptor; (Z), membrane depolarization. (See [color plate 3](#))

A large proportion of cholesterol delivered to mitochondria is converted to oxysterols: hydroxycholesterols and cerebrosterols (Pedersen *et al.*, 1989; Björkhem *et al.*, 1997). Two alternative pathways seem to operate. In one, hydroxylation by a P450 enzyme selectively expressed in brain, CYP46 (Lund *et al.*, 1999), produces the brain-enriched 24(S)-hydroxycholesterol or "cerebrosterol". This molecule is preferentially exported from the brain (Björkhem *et al.*, 1997, 1998). 24(S)-hydroxycholesterol is not a substrate for brain CYP7B, and is instead processed, in liver, by a dedicated hepatic 7 $\alpha$ -hydroxylase for 24(S)-hydroxycholesterol (CYP39A1; Li-Hawkins *et al.*, 2000), this enzyme appearing to be absent from brain. In the other, hydroxylation at the 25, 26/27 positions produces hydroxycholesterols that are, most likely, further hydroxylated at the 7 $\alpha$  position to provide

a series of di- (or tri-) hydroxycholesterols; these may be further metabolized to 3-oxo steroids (see Zhang *et al.*, 1997; Vatasery *et al.*, 1997). Note that the 27-hydroxylase (CYP27) is, like P450<sub>sc</sub>, mitochondrial in location (e.g., Okuda, 1994; Cali, 1999a), while the 24(S) hydroxylase is inferred to be microsomal (Lund *et al.*, 1999). Signaling pathways related to oxysterol metabolism are presented schematically in [Figure 15.2](#).

### **Do steroids target oxysterol sites?**

The possibility that sigma targets might define steroid binding sites (Jbilo *et al.*, 1997) was discussed earlier. Equally, some sterol targets might interact with steroids. Corticosterone, deoxycorticosterone, testosterone and progesterone are reported to competitively inhibit the binding of pharmacologic sigma agents (Su *et al.*, 1990) while effects of glucocorticoids on immunity could be, in part, via lymphocyte sigma targets (Deroca *et al.*, 1995). DHEA may also target sigma (Bergeron *et al.*, 1996; Debonnel *et al.*, 1996); Monnet *et al.* (1995) argued that DHEAsulfate is a sigma agonist, pregnenolone is an inverse agonist, while progesterone is an antagonist.

### **Is there cross-talk between sterol synthesis and surface ion-channels activity?**

While some pharmacologic agents act both at cell surface channels (e.g., GABA<sub>A</sub>) and intracellular sites, cross-talk may be more extensive. GABA<sub>A</sub> agonists, like PBR ligands, are reported to increase cholesterol conversion to pregnenolone in isolated rat retina GABA<sub>A</sub> agonists (Guarneri *et al.*, 1995). GABA channel activity can regulate intracellular sterol metabolism. Conversely, intracellular oxysterol sites can alter surface channel activity.

NMDA channels are modulated by DHEA. Potentiation of NMDA currents was reversed by either haloperidol or the selective sigma ligand NE100, arguing strongly that sigma occupancy can govern NMDA channel activity (Bergeron *et al.*, 1996; Debonnel *et al.*, 1996). The link between sigma ligands (including sigma-2 ligands; Couture and Debonnel, 1998) and NMDA currents raises the possibility that the effects of the important estrogen replacement analog equilin on NMDA currents (Brinton *et al.*, 1997) might be mediated, in part, through this pathway. Sigma ligands have also been reported to prevent depolarization neurotoxicity of retinal cells (Senda *et al.*, 1998) while, conversely, the sigma ligand trifluoperazine inhibits glutamate-induced mitochondrial depolarization (Hoyt *et al.*, 1997), reminiscent of the association of PBR with voltage-dependent anion channels of the outer mitochondrial membrane (McEnery *et al.*, 1992, 1993). In addition, sigma ligands can inhibit voltage-activated K<sup>+</sup> channels in intact neurons (Nguyen *et al.*, 1998) and in a neuropeptide positive tumor cell (Wilke *et al.*, 1999).

### **How might sterols modulate neuronal activity?**

There are four obvious routes. First, by direct interactions with cell surface channels. Neurotransmitter receptors contain steroid binding sites that modulate their activity, though it is unclear whether all such sites are structurally distinct from the transmembrane segment

that may interact with membrane cholesterol. Sterols could exert important influences via direct interactions with steroid and/ or cholesterol sites on neurotransmitter receptors.

Second, via DBI/endozapine. Modified cholesterol generally feed back on the enzymes involved in cholesterol synthesis, possibly including PBR/sigma. Feedback upon the production of the endogenous PBR ligand, DBI/endozapine, has not been excluded. This polypeptide targets GABA<sub>A</sub> in addition to PBR, and changes in DBI/endozapine activity or expression could govern the activity of GABA<sub>A</sub> and other DBI-responsive channels.

Third, via neurotransmitter release. Ligands targeting the sigma site and/or PBR are reported to modulate Ca<sup>2+</sup> release from intracellular stores (Vilner and Bowen, 2000; Hayashi *et al.*, 2000) and Ca<sup>2+</sup> fluxes (Ramp and Triggle, 1987; Church and Fletcher, 1995; Klette *et al.*, 1995; Brent *et al.*, 1996) and could alter synaptic firing properties.

Fourth, electrical coupling. Mitochondrial membrane polarization is tied to sterol metabolism, exemplified by the copurification of PBR with outer membrane porins (McEnery *et al.*, 1992, 1993). It is possible that cell-surface and intracellular membrane depolarization are electrically coupled.

#### *Local versus systemic control*

Cholesterol delivery and intracellular transport is crucial to the adrenal and gonadal synthesis of glucocorticoids, estrogens and androgens, and body homeostasis. Responsive changes in adrenal steroid levels may modulate memory encoding (Lathe, 2001). Corticosteroid abnormalities contribute to depression (Murphy, 1997) and estradiol promotes neuronal maintenance and can be beneficial in Alzheimer's disease (Honjo *et al.*, 1989; Haskell *et al.*, 1997; Asthana *et al.*, 1999).

### **Neuronal life and death: does local sterol metabolism govern apoptosis and neurodegeneration?**

Cholesterol depletion threatens cell integrity. It is not surprising, therefore, that there may be coupling between cholesterol synthesis and metabolism in the CNS, on the one hand, and cell growth and survival on the other. Further, imbalance between cholesterol and cell growth, such as may occur during disease states, seems to predispose to an active cell death process (e.g., apoptosis). It seems likely that brain could exploit cholesterol/apoptosis signaling mechanisms to achieve selective neuronal elimination during development; such processes may fail in ageing. Importantly, apoptosis may be governed at the local level by sterols, and at the systemic level by steroids. The different aspects are discussed separately below.

#### *Cholesterol metabolism and cell death*

Inhibition of cholesterol synthesis blocks DNA replication in primary brain-derived glial cultures (Langan and Volpe, 1987). This could not be overcome by exogenous cholesterol, suggesting an obligate requirement for intermediates in the cholesterol pathway (e.g., isoprenoids). Conversely, Michikawa and Yanagisawa (1999) report that blockade of



cholesterol production, but not that of isoprenoids, could result not merely in impaired cell division but in an active neuronal cell death process, such as apoptosis or necrosis.

As might be expected, agents targeting the intracellular sites discussed above (peripheral benzodiazepine receptor, PBR; the sigma/sterol isomerase site; and FK506 binding proteins involved in intracellular cholesterol trafficking) have all been linked to the regulation of apoptosis. PBR ligands can induce apoptosis in thymocytes (Tamimoto *et al.*, 1999; see also Hirsch *et al.*, 1998) but may be potent inhibitors of apoptosis in a human lymphoblastoid cell line (Bono *et al.*, 1999). Protoporphyrin IX, a natural sigma ligand, was found to potentiate cell death in energy stressed hepatocytes, whereas potentiation was blocked by cyclosporin A (Pastorino *et al.*, 1994).

Blockade of cholesterol synthesis by the sigma ligand haloperidol is cytotoxic to glioma cells (Vilner *et al.*, 1995) and to hippocampal neurons, possibly by necrosis rather than via apoptosis (Behl *et al.*, 1995). Conversely, benzodiazepines are protective for hippocampal neurons, though both GABA<sub>A</sub> and PBR were targeted (Schwartz-Bloom *et al.*, 2000). Haloperidol is also a dopamine receptor ligand, but specific dopamine agonists/antagonists were without effect (Vilner *et al.*, 1995). In lymphocytes, sigma ligands can exert immunosuppressive actions by preventing cholesterol synthesis and cell division (Jbilo *et al.*, 1997; Labit-Le Bouteiller *et al.*, 1998), possibly inducing apoptotic mechanisms (Brent *et al.*, 1996).

FK506 and cyclophilins may collaborate in cholesterol delivery to mitochondria and to cell surface microdomains (Dahlback-Sjoberg *et al.*, 1993; Uittenbogaard *et al.*, 1998). FK506 is protective against glutamate-mediated toxicity (Dawson *et al.*, 1993) and in a model of stroke (Sharkey and Butcher, 1994), while FK506 but not cyclosporin can block neuronal apoptosis in response to serum deprivation (Yardin *et al.*, 1998). The reverse was reported during hypoglycemic challenge (Ferrand-Drake *et al.*, 1999). Cyclosporin neurotoxicity on chronic administration (Hauben, 1996; Gijtenbeek *et al.*, 1999) may involve apoptotic mechanisms (McDonald *et al.*, 1996).

Together these reports argue that intracellular cholesterol metabolism, trafficking and mitochondrial delivery can govern the induction of apoptosis or necrosis. The major pathways involve the release, from mitochondria, of cytochrome C and apoptosis-inducing factor (AIF); these activate downstream death processes including caspase-mediated proteolysis (reviewed by Waters and Lavin, 1999). Agents interfering with mitochondrial integrity promote apoptosis—we should not forget that mitochondrial function is crucial to sterol/steroid synthesis.

#### *Steroids and cell death*

One of the best-studied apoptotic systems comprises the induction of apoptosis by glucocorticoids. In lymphocytes, glucocorticoids are powerful inducers of apoptosis and produce classical DNA fragmentation in CD4/CD8 lymphocytes that requires macromolecular synthesis. The process is inhibited by classical glucocorticoid antagonists such as RU486 and is increased by dexamethasone (Caron-Leslie *et al.*, 1991; LaVoie and Witorsch, 1995). However, different glucocorticoids differ markedly in their ability to promote apoptosis (Hofmann *et al.*, 1988) and the effect may not be mediated exclusively through a direct interaction with the nuclear glucocorticoid receptor GR.

In the brain, massive levels of apoptosis accompany developmental modeling during fetal growth (Blaschke *et al.*, 1996). In the adult, where neuronal division is restricted to proliferative regions in the dentate gyrus, olfactory bulb, and to a lesser extent in the cerebellum (Altman and Das, 1965), continuing proliferation is regulated systemically by adrenal steroids. Here both glucocorticoid excess and insufficiency can cause neuronal loss. In the rat, adrenalectomy results in cell death, particularly in the dentate gyrus, which can be prevented by glucocorticoid replacement (Sloviter *et al.*, 1989; Gould *et al.*, 1990). Neuronal loss is most pronounced in young animals but also takes place in later life (Sapolsky *et al.*, 1991) though only a proportion of animals showed cell loss (Jaarsma *et al.*, 1992). Conversely, excess glucocorticoids suppress cell proliferation (Gould *et al.*, 1992) and increase neuronal death; neurotoxic death in the hippocampus is reduced by adrenalectomy and aggravated by corticosterone (Sapolsky, 1985; reviewed by Sapolsky *et al.*, 1986; McEwen, 1999). Note, however, that glucocorticoid-driven neuronal cell death in the brain may not involve the classical apoptotic pathway (Masters *et al.*, 1989).

#### *Intracellular targets for apoptosis induction*

Both cholesterol depletion and glucocorticoid excess can promote cell death in the CNS. Is it possible that they operate through a common intracellular pathway? As noted previously, the sigma/EBP site related to cholesterol isomerase may have a steroidal ligand because corticosterone, deoxycorticosterone, testosterone and progesterone are reported to competitively inhibit the binding of pharmacologic sigma agents (Su *et al.*, 1990). The immunosuppressive effects of glucocorticoids may be mediated, in part, through an interaction with the sigma receptor (e.g., Derocq *et al.*, 1995) while glucocorticoids and oxysterols (see below) synergise to promote lymphocyte apoptosis (Thompson *et al.*, 1999).

Is apoptosis positively regulated? In the above it has been suggested that apoptosis is brought about passively, by disruption of orderly cholesterol trafficking and metabolism in the cell. A contrasting view is emerging, in which apoptotic mechanisms may be deliberately activated *in vivo* by steroidal molecules. Specifically, some oxidised derivatives of cholesterol are extremely potent inducers of cell death.

Cholesterol itself is largely inert, but when oxidised at the 25-position, and more particularly at the 7-position, it gives rise to a series of highly efficient inducers of apoptosis in the immune system, endothelial cells, and in the CNS. The 7-position modification is intriguing, particularly in view of the fact that neurosteroids can be selectively modified at the 7-position in brain extracts (see [Box 15.6](#)) and 7-modified cholesterol (and 7-oxo molecules) are detected in primary brain extracts (Vatassery *et al.*, 1997). Note, however, that the data are often confusing, not only because cholesterol may be further metabolized during assay, but also because, at least in theory, either excess or depletion could cause similar effects if there is significant feedback regulation.

In lymphocytes, 25-hydroxycholesterol and 7 $\beta$ ,25-dihydroxycholesterol diminish mitogen-induced proliferation (Richert *et al.*, 1986), block HIV replication (Moog *et al.*, 1998) and induce the death of lymphoma cells and thymocytes (Christ *et al.*, 1993). 7 $\beta$ -hydroxy and 7-oxocholesterols induce apoptosis in lymphocytes (Lizard *et al.*, 1997, 1998) and synergize with glucocorticoids for immunosuppression and apoptosis induction (Johnson

*et al.*, 1997). 7-oxocholesterol causes typical DNA fragmentation in human and bovine endothelial cells (Lizard *et al.*, 1997) while in murine tumor lines cell-death mediated by either 7-oxocholesterol or 25-hydroxycholesterol was prevented by inhibitors of macromolecular synthesis (Hwang, 1992) suggestive of classic apoptosis. 7 $\beta$ - and 7-oxocholesterols elicit necrosis in fibroblasts (Lizard *et al.*, 1999) while, in the brain, 24-hydroxycholesterol and cholestanol (5 $\alpha$ -cholestan-3 $\beta$ -ol) are neurotoxic (Kolsch *et al.*, 1999) and 7 $\beta$ - and 7-oxocholesterols are reported to kill cultured cerebellar granule cells (Chiang and Liu, 1998).

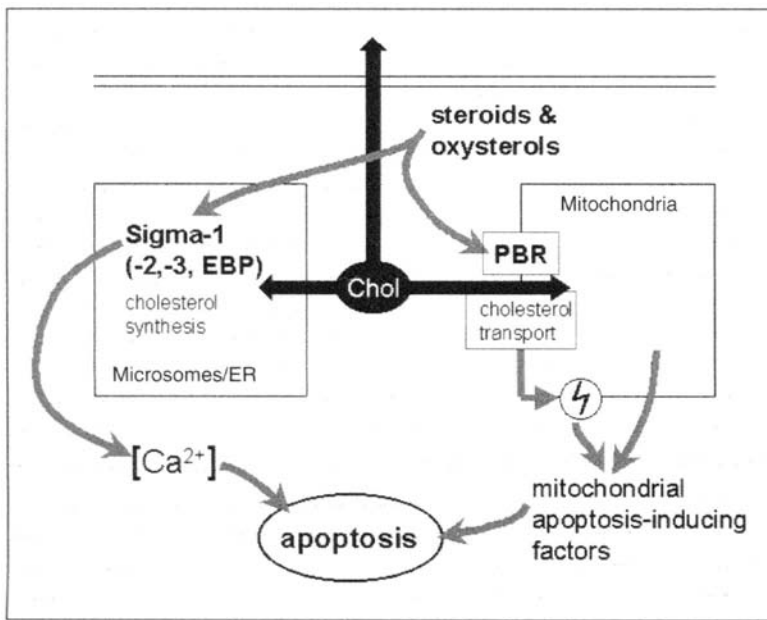
The nature and stereochemistry of the 7-position modification appears to be of critical importance. Cell killing mediated by 25-hydroxycholesterol can be abrogated by upregulation of 7 $\alpha$ -hydroxylase systems (Leighton *et al.*, 1991) suggesting that 7 $\alpha$ -hydroxysterols are inert or protective. 7 $\beta$ -hydroxy and 7-oxo molecules efficiently induce apoptosis in endothelial cells (Lemaire *et al.*, 1998) and lymphocytes (Zhang *et al.*, 1997), but the 7 $\alpha$ -hydroxy molecules failed to produce cell death and may be protective against apoptosis (Zhang *et al.*, 1997). In a particularly interesting study, Chisholm and colleagues purified a cholesterol derivative that appears to be an exceptionally potent inducer of apoptosis. Low density lipoprotein (LDL) circulates in the bloodstream as a cholesterol complex and oxidized LDL is itself a potent inducer of apoptosis (Nishio *et al.*, 1996; Sata *et al.*, 1998). Chisolm *et al.* (1994) extracted and purified the toxic activity associated with oxidized LDL. Perhaps surprisingly, the primary active molecule was 7 $\beta$ -hydroperoxy cholesterol (7 $\beta$ -OOH-cholesterol). This molecule is a major non-enzymic oxidation product of cholesterol (see Chisholm *et al.*, 1994; Beriozov *et al.*, 1990) though oxidation at other positions (e.g., 25-hydroxylation) is also likely to occur. 7 $\beta$ -OOH-cholesterol has been observed *in vivo* and concentrations increase with age (Ozawa *et al.*, 1991). One potentially confusing factor is that a proportion of 7 $\beta$ -OOH-cholesterol is likely to derive from the diet, possibly arguing against a specific *in vivo* signaling role, though a possible role of 7 $\alpha$  derivatives produced *in vivo* could be to compete with environmental or damage-induced 7 $\beta$ -modified cholesterols.

#### *Receptors for apoptotic oxysterols*

As remarked earlier, passive interference with cholesterol metabolism predisposes to apoptotic processes. This we feel contrasts with the potent induction of apoptosis by molecules such as 7 $\beta$ -OOH-cholesterol. Although it is possible that 7 $\beta$ -OOH-cholesterol and related molecules such as 7 $\beta$ -hydroxycholesterol merely inhibit cholesterol metabolism and trafficking, they may also have dedicated receptors.

At the level of cholesterol synthesis, 7 $\alpha$ -hydroxylation of cholesterol was required for repression of cholesterol synthesis in one study (Axelson *et al.*, 1996) although 3-oxo and 7-oxo molecules were also effective. In contrast, Martin *et al.* (1997) report that 7 $\alpha$ -hydroxycholesterol fails to inhibit cholesterol synthesis, perhaps suggesting that, as for apoptosis induction, 7 $\beta$ -hydroxycholesterol is the active molecule that blocks cholesterol synthesis and ultimately elicits cell death. However, the identity of the cholesterol derivatives that feedback on the enzymes involved in cholesterol synthesis remains to be established.

Oxysterols are also likely to target nuclear receptors to repress transcription of genes involved in cholesterol synthesis. In addition to reducing proteolytic activation of the sterol response element (SRE) binding protein SREBP (Brown and Goldstein, 1997; Lund *et al.*, 1998), there may be dedicated nuclear receptors responding to 7-modified cholesterol and bile acids. LXR $\alpha$  responds principally to oxysterols (Janowski *et al.*, 1996), particularly 24 (S)-hydroxycholesterols (Lehmann *et al.*, 1997), as does the inhibitory receptor CAR- $\beta$  (Forman *et al.*, 1998), possibly FXR (Makashima *et al.*, 1999; Parks *et al.*, 1999) and potentially PPAR, PXR, SXR and CPF, nuclear receptors regulating peroxisome proliferation and xenobiotic metabolism (Blumberg *et al.*, 1998; Steir *et al.*, 1998; Waxman, 1999) or the gene encoding hepatic cholesterol 7 $\alpha$ -hydroxylase (Nitta *et al.*, 1999). At the cell surface, they may target a receptor that modulates cAMP production (Moog *et al.*, 1991), potentially similar to the brain adenylyl cyclase that is regulated by calcineurin, FK506 and cyclosporin (Antoni *et al.*, 1998).



**Figure 15.3 Possible convergence of oxysterol and steroid signaling pathways on apoptosis induction.**

The field remains confused. That cholesterol metabolism is crucial to mitochondrial integrity, surface neurotransmitter receptor activity, and cell survival, is beyond any reasonable doubt. That 7-modified cholesterol can be either protective (e.g., 7 $\alpha$  molecules) or toxic (7 $\beta$ -hydroxy- and 7 $\beta$ -hydroperoxy-cholesterols) is also well-established. But we have little idea at what level this regulation occurs, or at which receptor the protective and destructive oxysterols compete for binding (if indeed they do). Are there intracellular or cell surface ion channels that are directly modulated by these molecules? Is mitochondrial energy generation subject to sterol regulation? The pathways might converge

on the endogenous benzodiazepine, DBI/endozapine, to link, for example, GABA receptor function with mitochondrial steroid transport and metabolism. Mitochondrial membrane potential and levels of free  $\text{Ca}^{2+}$  are important regulators of apoptosis (Crompton *et al.*, 1999). Apoptosis induction may operate, in part, by sterol-regulation of  $\text{Ca}^{2+}$  levels, evinced by the fact that ligands targeting sigma (Church and Fletcher, 1995; Klette *et al.*, 1995; Brent *et al.*, 1996) or PBR (e.g., Ramp and Triggle, 1987) can affect  $\text{Ca}^{2+}$  flux. PBR appears to bind to ion channels of the mitochondrial membrane (McEnery *et al.*, 1992, 1993); mitochondrial membrane potential and apoptosis initiation may be subservient to sterol binding at PBR (Figure 15.3).

Intracellular regulation of oxysterol metabolism and apoptosis, both centrally dependent on the mitochondrion, are paralleled at the systemic level by the regulation of apoptosis by glucocorticoids—the synthesis of glucocorticoids (and mineralocorticoids) only takes place in mitochondria. Is it possible that steroids (acting systemically), and hydroxycholesterols (local intracellular action), once targeted the same intracellular receptor or receptors? In which case one must consider the possibility that neurosteroids may be evolutionary relics, performing local intercellular signaling but targeting intracellular receptors.

### BOX 15.6

#### STEROID/STEROL 6 AND 7 HYDROXYLATION

There is an ongoing debate concerning the enzymology and role of steroid and sterol hydroxylation in brain. Pregnenolone and DHEA, either produced by local synthesis or derived from the circulation, have alternative metabolic routes. On the one hand, via  $3\beta$ -HSD to progesterone and androstenedione; on the other, hydroxylation at the 7 (or 6) positions is perhaps the major metabolic route for these molecules in brain extracts (Akwa *et al.*, 1991). In rat and mouse brain,  $3\beta$ -hydroxy steroids including pregnenolone, DHEA and  $5\alpha$ -androstane- $3\beta$ ,  $17\beta$ -diol (A/ane-diol) are hydroxylated at the  $6\alpha$ ,  $7\alpha$  and  $7\beta$  positions, while 7-hydroxylated cholesterol are also produced (see text). Steroid and sterol hydroxylation at the 7 position has profound effects on receptor activation.  $7\alpha$ -hydroxylation of DHEA reduces its affinity for the estrogen receptor (Li *et al.*, 1978) while other  $7\alpha$ -substituted steroids are particularly potent modulators of androgen, estrogen or aldosterone action (Weier and Hofmann, 1975; Bullock *et al.*, 1978; deFriend *et al.*, 1994; Beri *et al.*, 1997).  $7\alpha$ -hydroxypregnenolone is a major steroid in some teleost fish (Ponthier *et al.*, 1998). These reports point to early evolutionary ancestry and conservation of a modulatory site with  $7\alpha$ -stereochemistry.

**Enzymology.** It is so far unclear whether one or several enzymes are responsible for 6- and 7-hydroxylation of steroids, including DHEA, A/ane diol and A/ene diol, and cholesterol. Akwa *et al.* (1991, 1992) reported a brain activity converting pregnenolone and DHEA to  $7\alpha$ -hydroxy derivatives. Little  $7\beta$ -hydroxy steroid was produced and the reaction was sensitive to estradiol. Warner *et al.* (1989) reported that brain and prostate catalyze the conversion of  $5\alpha$ -androstane- $3\beta$ ,  $17\beta$ -diol to the triol compound, with most hydroxylation at the  $7\beta$ -position, significant modification at  $7\alpha$ , and lesser conversion at  $6\alpha$ . Stromstedt *et al.* (1993) argued in favor of a single enzyme activity, and that this brain enzyme also participates in the hydroxylation of  $3\beta$ -hydroxy- $5\alpha$ -pregnan-20-one (Figure 15.1: Isopregnanolone;  $3\beta$ -hydroxy- $5\alpha$ -dihydroprogesterone).  $3\alpha$  versions were

not modified. Gemzik *et al.* (1992a, b, c) report that conversion of 5 $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol to the 7 $\beta$ -, 7 $\alpha$ - and 6 $\alpha$ -hydroxylated molecules by rodent prostate is equally reduced by P450 inhibitors, arguing that one enzyme catalyzes all three hydroxylations in this tissue. Parallel studies in primates indicate that different enzymes mediate 6- and 7-hydroxylation (Gemzik *et al.*, 1992b). In contrast, on the basis of inhibitor studies, Morfin and colleagues (Doostzadeh and Morfin, 1997; Doostzadeh *et al.*, 1997) suggest that distinct brain enzymes are responsible for 7 $\alpha$ - and 7 $\beta$ -hydroxylation. These authors suggest that 7 $\alpha$ -hydroxylation of pregnenolone and  $\Delta^5$ -androstenediol in brain may be performed by one enzyme, while DHEA is hydroxylated by another.

We reported a P450 cDNA, CYP7B, encoding an enzyme that selectively 7 $\alpha$ -hydroxylates DHEA, pregnenolone and  $\Delta^5$ -androstenediol (Stapleton *et al.*, 1995; Rose *et al.*, 1997). The enzyme is also active against hydroxycholesterol and is likely to be identical to hepatic oxysterol-selective 7 $\alpha$ -hydroxylase (Schwarz *et al.*, 1997), an enzyme distinct from the major hepatic cholesterol 7 $\alpha$ -hydroxylase (CYP7A). The CYP7B enzyme thus most resembles the activity reported by Akwa (1992) while the rat prostate activity could represent a third enzyme. Breast tumor and ovarian cells are particularly rich in an oxysterol 7-hydroxylation activity (Couch *et al.*, 1975; Li *et al.*, 1978; Payne *et al.*, 1995) although the enzyme described by Payne *et al.* (1995) may be distinct from both CYP7A and 7B as it is not competitively inhibited by DHEA. Note that CYP46, which hydroxylates cholesterol at 24 (S), is abundantly expressed in brain (Lund *et al.*, 1999). Activity on non-cholesterol substrates has not yet been evaluated.

**7-hydroxylation of cholesterol and oxysterols.** In liver, cholesterol hydroxylation at the 7 $\alpha$  position (by CYP7A) is a major route for metabolic elimination via conversion to bile acids and subsequent secretion. In brain, however, the major route for cholesterol metabolism is to 24 (S)-hydroxycholesterol that is secreted from brain, while 25- and 27-hydroxycholesteroles are hydroxylated in brain, as in liver, at the 7 $\alpha$  position (see text). Hepatic cholesterol 7 $\alpha$ -hydroxylase CYP7A is not expressed in brain, and oxysterol 7 $\alpha$ -hydroxylation is likely to be mediated by CYP7B (Rose *et al.*, 1997; Schwarz *et al.*, 1997; Martin *et al.*, 1997). Hydroxylation is unlikely to be the end of the metabolic pathway, for a distinct hepatic 7 $\alpha$ -hydroxycholesterol dehydrogenase/oxidoreductase has been reported with significant homology to 11 $\beta$ -hydroxysteroid dehydrogenases (Song *et al.*, 1998; see Box 15.5). A patient deficient in CYP7B activity has been reported (Setchell *et al.*, 1998); the genetic lesion precipitated the accumulation of hepatotoxic unsaturated monohydroxy bile acids and oxysterols, and liver failure. The severity of the disease precluded brain function analysis.

**Does 7-hydroxylation gate receptor access?** Cholesterols, and particularly 7 $\beta$ -oxidised cholesterols, are inducers of apoptosis (see text), 7 $\alpha$ -oxidised cholesterols are most probably protective, and although the specific receptors responsible are not known, sigma/EBP and PBR furnish likely candidates. In other words, 7 $\alpha$  versus 7 $\beta$  modification gates sterol access to the pertinent target. Steroid signaling is thought to have evolved from sterol signaling, and we suggest (see text) that DHEA and its 7 $\alpha$ -hydroxylated derivatives might modulate glucocorticoid action at cognate receptors. However, whether the action is via GR, via another nuclear receptor type (LXR, FXR, PXR), or (perhaps more likely) at intracellular sites also targeted by glucocorticoids, remains to be determined. Recent reports suggest that oxysterols may have one or more dedicated receptors distinct from the classic hormone-binding nuclear receptors (Janowski *et al.*, 1996; Forman *et al.*, 1998; Parks *et al.*, 1999; Makishima *et al.*, 1999); the same may be true for 6 or 7-hydroxylated pregnansteroids. There is some evidence that 7-modification

may gate activation of the estrogen receptor (and perhaps the androgen receptor). 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (see Figure 15.1) is a breakdown product of testosterone and is a significant agonist of estradiol and androgen action at their cognate receptors (ER and AR) in breast cancer cells (Hackenberg *et al.*, 1993). Hydroxylation of this molecule, by an activity that modifies both DHEA and  $\Delta^5$ -androstenediol (Li *et al.*, 1978), and catalyzed by CYP7B (Rose *et al.*, 1987), abolishes activity at ER (Li *et al.*, 1978). Similarly, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol and DHEA were both active in stimulating AR in prostate cancer cells, but 7-oxoDHEA (potentially inter-converting with 7-hydroxy derivatives) was ineffective (Miyamoto *et al.*, 1998). Note the effect of 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol may involve separate stimulatory and inhibitory components (Boccuzzi *et al.*, 1994). 7-hydroxylated derivatives of DHEA have further potential as local modulators of aromatase.

In support of a regulatory role for 7-hydroxylation, Akwa *et al.* (1993) report that alternative metabolism of pregnenolone and DHEA to either progesterone and androstenedione (by 3 $\beta$ -HSD) or to 7 $\alpha$ -hydroxylated derivatives (possibly by CYP7B) is dependent on the plating density of purified type 1 astrocytes *in vitro*. At low plating density 3 $\beta$ -HSD metabolites predominated and only 1% of substrate was converted to 7 $\alpha$  derivatives. In marked contrast, at high plating density 50% of substrate was converted to 7 $\alpha$ -hydroxy products while 3 $\beta$ -HSD metabolites were scarce (Akwa *et al.*, 1993). There may be active switching between the two pathways.

#### OXYSTEROLS AND NEUROSTEROIDS: IS THERE A LINK WITH AGEING?

Steroids and sterols contribute to the regulation of neuronal activity and cell death in the CNS. This opens the possibility that deregulated steroid/sterol signaling might accompany or cause cognitive decline with ageing. Sapolsky and colleagues (1986) proposed that the slow rise in glucocorticoids that accompanies ageing might precipitate a cascade of neurodegeneration, further amplified through increasing failure of the brain to repress adrenal output. In its simplest form the hypothesis has not been fully corroborated, giving way to a reciprocal contention that the effects of glucocorticoids might be exacerbated by concomitant decline in another adrenal steroid, dehydroepiandrosterone (DHEA).

DHEA is the major adrenal steroid in primates, though not in rodents (see earlier). As widely discussed, plasma levels of free DHEA in human decay asymptotically but variably over the lifetime of human individuals, while similar declines are observed in other primates, paralleling their lifespans (see Orentreich *et al.*, 1992; Sapolsky *et al.*, 1993). The possibility that DHEA decline contributes to age-related diseases has been debated (Morales *et al.*, 1994; Nestler, 1996; Hinson and Raven, 1999); encouraging effects of high dose DHEA replacement therapy have been recorded in early old age (Morales *et al.*, 1994) but not in the more elderly (Wolf *et al.*, 1997, 1998). DHEA had at best marginal efficacy in an extended trial (Baulieu *et al.*, 2000) and there was no correlation between cognitive status or decline with steroid levels (Moffat *et al.*, 2000).

Other related molecules also decline markedly with age, and possibly both in rodents and humans. These include the precursor molecule 7-dehydrocholesterol in both sciatic nerve and skin (MacLaughlin and Holick, 1985; De Groot, 1989; Bourré *et al.*, 1990), plasma levels

of 24(S)-hydroxycholesterol (Lütjohann *et al.*, 1996, 1998) and skin vitamins D<sub>3</sub> (MacLaughlin and Holick, 1985), though others have suggested that 24(S)-hydroxycholesterol levels in brain (rather than plasma) may increase with age, at least in mice (Lund *et al.*, 1999). Perhaps more likely is that conversion and transport at the sterol/steroid frontier display general age-related loss of function, leading one to suspect that elevated blood DHEA in primates might reach intracellular targets to offset this decline.

The reciprocal decline in DHEA and slow increase in glucocorticoid levels does seem to correlate with age-related cognitive dysfunction, but the relationship between cortisol/DHEA (and pregnenolone) levels and ageing is not as clear-cut as has sometimes been asserted (e.g., Kalmijn *et al.*, 1986). A confounding factor is that DHEA and pregnenolone levels may be significantly elevated or reduced in brain disease, including anxiety and chronic fatigue syndrome (George *et al.*, 1994; Takebayashi *et al.*, 1998; Heuser *et al.*, 1998; Scott *et al.*, 1999; De Becker *et al.*, 1999) in addition to the well-documented increases in cortisol production that can accompany depression (reviewed by Murphy, 1991; Sapse, 1997). What also emerges from these studies is that the absolute level of DHEA is perhaps less important than the DHEA/cortisol ratio, contributing to the idea that DHEA might be a "natural" antiglucocorticoid.

Circumstantially the evidence is strong. Excess glucocorticoids impair learning and memory, while DHEA and pregnenolone improve post-training memory when injected into limbic structures of the mouse brain (Flood and Roberts, 1988; Flood *et al.*, 1992, 1995), and enhance synaptic plasticity (e.g., Yoo *et al.*, 1996). DHEA can have antiglucocorticoid actions both *in vitro* (May *et al.*, 1990; Araneo *et al.*, 1990, 1995; Blauer *et al.*, 1991) and *in vivo* (Daynes *et al.*, 1990), including antiglucocorticoid effects in the CNS (Singh *et al.*, 1994; Fleshner *et al.*, 1995; reviewed by Kalimi *et al.*, 1994). Inhibition of astrocyte proliferation by the synthetic glucocorticoid dexamethasone was reversed by DHEA as well as progesterone (Crossin *et al.*, 1997) and DHEA enhancement of hippocampal primed-burst potentiation was blocked by stress (Diamond *et al.*, 1999). Kimonides *et al.* (1999) report that DHEA can protect hippocampal neurons against cortico-sterone-mediated neurotoxicity while corticosterone-induced nuclear accumulation of GR in a mouse hippocampal cell line was blocked by DHEA (Cardounel *et al.*, 1999). DHEA is also reported to block corticosterone inhibition of synaptic potentiation in dentate gyrus (Kaminska *et al.*, 2000). In other tissues, McIntosh *et al.* (1999) report systemic antiglucocorticoid effects of DHEA on hepatic lipogenesis, thymic and spleen weight reduction and serum IGF-1. Nevertheless, a direct effect on GR seems unlikely, although changes in the subcellular distribution of GR in response to DHEA have been noted (Cardounel *et al.*, 1999). The antiglucocorticoid activity of DHEA could not be reconstituted in an *in vitro* system (Rupprecht *et al.*, 1996) and we have confirmed that DHEA is, at best, a poor inhibitor of human GR (unpublished observations). Two explanations may be offered.

First, DHEA may act systemically. DHEA can reduce cortisol levels in healthy adult volunteers (Wolf *et al.*, 1997) and serum corticosterone levels in rats, possibly by altering peripheral glucocorticoid metabolism (Homma *et al.*, 1998). DHEA can also act at central GABA<sub>A</sub> and possibly PBR and sigma sites; free DHEA could thereby feed back to the brain to alter adrenal output. In support, the central GABA<sub>A</sub> agonist alprazolam decreased plasma cortisol but increased DHEA (Krobeth *et al.*, 1999) and systemic sigma agents affect



glucocorticoid (and no doubt DHEA) release from the adrenal (Pechnick and Poland, 1989; Matheson *et al.*, 1991; Gudelsky and Nash, 1992; Eaton *et al.*, 1992).

Second, local DHEA metabolism may be required for activity (Shealy *et al.*, 1995), particularly in the immune system (Morfin and Courchay, 1994). As we discussed (Box 15.6), 7-hydroxylation is the major route for the metabolism of DHEA in the CNS. In lymphocytes, 7 $\beta$ -hydroxylated steroids (including androstenediol: 5-androstene-3 $\beta$ , 7 $\beta$ , 17 $\beta$ -triol) are reported to activate the immune response in mice (Padgett and Loria, 1994; Loria *et al.*, 1996) and 7 $\beta$ -androstenediol molecules may counteract corticosteroid-induced immunosuppression *in vitro* (Loria, 1997). Others have argued that DHEA exerts its immunomodulatory activity not via 7 $\beta$ -modified derivatives but instead through 7 $\alpha$ -hydroxylation (Lafaye *et al.*, 1999), a conversion taking place in brain (Rose *et al.*, 1997) (though binding of hydroxylated derivatives of androstenediol and DHEA to the same cellular targets has not been formally demonstrated). 7 $\alpha$ -hydroxyDHEA was more effective than DHEA in preventing dexamethasone-induced thymic cell loss (Chmielewski *et al.*, 2000); 7-oxoDHEA had beneficial effects on long-term memory retention in old (22 month) mice, as did 7 $\alpha$ -hydroxyDHEA, while DHEA was less effective (Shi *et al.*, 2000). 7-oxoDHEA may though interconvert with 7-hydroxylated derivatives.

Does DHEA act as an antiglucocorticoid at the level of steroidogenesis and/or apoptosis? Glucocorticoids (corticosterone and deoxycorticosterone) and related steroids are active at the sigma/sterol site (Su *et al.*, 1990; Derocq *et al.*, 1995) as are DHEA and pregnenolone (Monnet *et al.*, 1995; Debonnel *et al.*, 1996), with EBP and PBR affording further promising co-targets. DHEA was effective in preventing dexamethasone-induced apoptosis in mouse thymocytes (Chmielewski *et al.*, 2000). However, a specific antagonism between DHEA and glucocorticoids at sigma, EBP or PBR has not been investigated. Notwithstanding, one might reasonably speculate that DHEA and its derivatives, particularly 7 $\alpha$ -hydroxylated DHEA, might act as protective oxysterol analogues by protecting, e.g., sigma or EBP from glucocorticoid inhibition. Steroids like DHEA and its derivatives could have an advantage over endogenous sterols because, unlike native oxysterols, they cannot be side-chain hydroxylated—this could prevent further metabolism and export.

This challenges the assumption that glucocorticoids and antiglucocorticoids principally act through the nuclear glucocorticoid receptors (GR and MR), and emphasizes the importance of non-nuclear targets including sigma, EBP and PBR. One must however avoid the impression that there is an either/or choice to be made between nuclear receptors (GR/MR) and sterol metabolism sites (PBR/ sigma/EBP). On the one hand, the nuclear receptors are very likely to play a prominent role outside the nucleus, and possibly at the cell surface (see mGR, earlier). On the other, there are many other nuclear receptors that may respond to steroids important in this discussion (e.g., glucocorticoids and DHEA). This latter possibility is amply illustrated by studies on the bioactivity of rifampicin.

#### A CONFUSING DIVERSITY OF NUCLEAR RECEPTORS: THE RIFAMPICIN EXAMPLE

Rifampicin (rifampin) is a powerful antibacterial antibiotic used in peripheral and brain infections because the drug enters the brain. However, it interacts adversely with

corticosteroid therapy (e.g., Jubiz and Meikle, 1979), attributed to rifampicin-induced alterations in hepatic corticosteroid metabolism (Edwards *et al.*, 1974) and expression of P450s including those metabolizing bile acids (Weitholz *et al.*, 1996). Recently, rifampicin was reported to activate directly the human glucocorticoid receptor (GR) *in vivo* (Calleja *et al.*, 1998). This was contradicted by another study (Jaffuel *et al.*, 1999) and it seems that rifampicin may operate through a different receptor.

Hepatic CYP expression is controlled by a group of nuclear receptors that include PXR (pregnan-X receptor), CAR $\beta$ , PPAR (peroxisome proliferator receptors), LXR (liver-X (oxysterol) receptor), FXR (farnesol/oxysterol receptor), and the probable oxysterol receptors SXR and CPF (reviewed, in part, by Waxman, 1999). Many of these (if not all) are well expressed in brain. Although an interaction with the vitamin D receptor was suggested (Mellon, 1984), it transpires that PXR mediates rifampicin induction of liver P450 CYP3A4 (Bertilsson *et al.*, 1998; Goodwin *et al.*, 1999) as well as induction by other xenobiotics. Confusingly, all these receptors and the pathways they govern interact in diverse ways. For instance, PPAR can activate an estradiol-responsive gene (Nunez *et al.*, 1997). The glucocorticoid agonist dexamethasone increases PXR-1 mRNA in rat (Zhang *et al.*, 1999) and, depending on species, PXR is potently activated by glucocorticoids and antiglucocorticoids including the powerful glucocorticoid antagonist pregnenolone-16 $\alpha$ -carbonitrile (Kliwer *et al.*, 1998). It is reasonable to suppose that modulation of gene action by neurosteroids including pregnenolone, DHEA and their derivatives may be partly mediated through these nuclear receptors, in addition to other direct actions on cell-surface, cytoplasmic and mitochondrial receptors. Finally, if both ER and GR have cell-surface forms, this may be a common feature of nuclear receptors; PXR for example could also have a non-nuclear isoform that can respond to glucocorticoids and antiglucocorticoids.

#### DEVELOPMENT, STEROL SIGNALING AND SUBCELLULAR TARGETING

Brain function is critically reliant on sterol metabolism. It comes as no surprise that sterol metabolism in the brain, an organ whose signature is plastic remodeling in response to experience, is also critical for brain development. Abnormalities of sterol metabolism predispose to developmental deficits and there is a strong association between fetal malformations and cholesterol synthesis perturbations during pregnancy. Inborn errors in early cholesterol metabolism, including Smith-Lemli-Opitz syndrome (7-dehydrocholesterol reductase), Niemann-Pick disease type C (NPC1-mediated sterol transport) and cerebrotendinous xanthomatosis (cholesterol 27-hydroxylase), discussed earlier, induce pronounced developmental abnormalities in addition to mental and neurologic complications. Knockout mice for ApoB have defects in brain development (Farese *et al.*, 1995; Huang *et al.*, 1995; Homanics *et al.*, 1995), while mice lacking the ApoB receptor megalin, a member of the LDLR superfamily, show defects akin to Smith-Lemli-Opitz (Willnow *et al.*, 1996).

One may infer that cholesterol traffic is critical for both structural and informational processes. Structurally, in the synthesis of brain membranes (for instance during postnatal myelination of brain neurons), and informationally, for orderly developmental selection of

neuronal circuits (exploiting oxysterol/apoptosis pathways to eliminate inappropriately wired neurons). However, recent evidence suggests that sterols (and possibly steroids) may play a far more direct role in pattern formation during brain development. This is suggested by two discoveries—that the product of the key developmental regulatory gene *Hedgehog/Sonic hedgehog* (*Hh/Shh*) is covalently linked to cholesterol, and that the mammalian homologue of the developmental gene *Fushi tarazu* of *Drosophila melanogaster* is, intriguingly, a regulator of sterol metabolism.

The fruitfly *Hh* protein undergoes autocatalytic cleavage resulting in the addition of cholesterol, via its  $3\beta$ -OH group, to the C-terminus of the N-terminal fragment (Porter *et al.*, 1996), very substantially increasing its effectiveness as a signaling molecule. Cholesterol linkage is not restricted to *Hh*, and it is not clear whether cholesterol is the only adduct (Porter *et al.*, 1996). In *Drosophila*, unlike mammals, yeast-derived ergosterol is the major membrane sterol. Studies with the mammalian *Hh* homolog (*Sonic hedgehog*, *Shh*) have shown that *Shh* protein can be coupled efficiently to other sterols including desmosterol and 7-dehydrocholesterol *in vitro* (Cooper *et al.*, 1998).  $3\beta$ -hydroxy steroids were not tested. In flies, cholesterol-linked *Hh* protein modulates gene expression via *Patched* (*Ptc*). This protein has similarities to NPC1 protein implicated in Niemann-Pick disease type C and contains a sterol sensing domain. *Hh* (and by inference *Shh*) may therefore be involved in sterol signaling (Johnson and Scott, 1998).

Disruption of *Shh* in humans is responsible for holopresencephaly (Belloni *et al.*, 1996; Roessler *et al.*, 1996), a usually lethal disease of variable penetrance whose consequences range from microcephaly to failure of hemispheric separation and craniofacial abnormalities. A similar condition is produced by disruption of the mouse *Shh* gene (Chiang *et al.*, 1996). It is of note that Smith-Lemli-Opitz, discussed earlier, is accompanied by malformations similar to holopresencephaly. Together these observations argue that sterol signaling mediated by *Shh* is critical to brain development. NPC1, a vertebrate analog of the *Hh*-reponsive *Ptc* (Johnson and Scott, 1998), is involved in sterol trafficking to and from cell surface lipoprotein receptors, particularly the LDL receptor, and it is intriguing that a novel LDL-receptor family member shows homology to the “neuropeptide head regulator” of Hydra (Hermans-Borgmeyer *et al.*, 1998). Control of development by sterol (rather than steroid) signaling between cells may have arisen early in evolution.

A clue to the nature of this sterol signaling was provided by studies on the *Drosophila* mutant *Fushi tarazu* (*Ftz*). The *Ftz* gene encodes an important homeobox gene that determines segmentation patterns during fly development. Molecular cloning of the fruitfly receptor for *Ftz* protein identified *FtzFl* (Guichet *et al.*, 1997; Yu *et al.*, 1997), an orphan receptor of the steroid nuclear receptor superfamily. This would be purely circumstantial, except that, most remarkably, CPF (a human homolog of *Ftz-Fl*) has been demonstrated to regulate cholesterol  $7\alpha$ -hydroxylase (CYP7A) expression in liver (Nitta *et al.*, 1999).

From an evolutionary perspective, it has been argued that the *Ftz* system was first required for nervous system development (where sterol regulation would make sense given the enormous brain demand for structural cholesterol) and only more recently for developmental specification, as exemplified by its role in *Drosophila* segmentation (Brown *et al.*, 1994).

Analysis of *Drosophila* signaling has provided a final insight into the mammalian system. Signaling by cholesterol-bound Hh can extend over several cell diameters, arguing that there must be a mechanism that facilitates transport of Hh protein to more distant sites. Such a function is provided by the product of the *Drosophila* gene *Tout-velu*, the mammalian homolog of which is a transmembrane protein of the endoplasmic reticulum (reviewed by Strigini and Cohen, 1999). While not proven, these reports begin to suggest that Hh protein, and by inference Shh, can traffic from the interior of the cell to sites some distance away, and in particular may concentrate in cholesterol-rich rafts in distal membranes (Rietvald *et al.*, 1999). These are principal sites for GPI-linked protein accumulation and for signal transduction; it is possible that local transport of Hh exploits a sterol transport mechanism (e.g., that comprising caveolin and FK506- and cyclosporin-binding proteins; Uittenbogaard *et al.*, 1998).

If sterol ligands modulate CPF activity, as seems likely in view of the sterol-responsiveness of the CYP7A gene, we have a remarkable situation in which a sterol-binding nuclear receptor interacts with a key homeobox developmental regulator to control gene expression. Some homeobox proteins, e.g., Antennapedia, can penetrate the cell surface by an unusual receptor and temperature-independent mechanism to reach the nucleus (Joliot *et al.*, 1991; Derossi *et al.*, 1996). Because homeobox protein-mediated transport cotransfers ligands bound to the homeoprotein (Theodore *et al.*, 1995) it is plausible to suggest that some nuclear receptors, at least, traffic from the cell surface to the nucleus, and perhaps (like Shh) even between cells (discussed by Prochiantz and Theodore, 1995). Whether similar overlaps in developmental and physiological regulation exist for other nuclear hormone receptors (GR? ER?) remains to be elucidated, but the appearance of immunologically related versions of GR and ER at the cell surface (Gametchu *et al.*, 1999; Monje and Boland, 1999) might be consistent with such a process. The intimate involvement of the homeo domain proteins Pbx and Oct-1 in glucocorticoid repression via a glucocorticoid response element (Subramaniam *et al.*, 1998) is most suggestive, particularly because it has been suggested that Pbx1 and GR proteins may form a tight complex (see Subramaniam *et al.*, 1999) and both GR and the progesterone receptor have been shown to associate tightly with FKBP52 implicated in intracellular transport (Schmitt *et al.*, 1993; Smith *et al.*, 1993; Silverstein *et al.*, 1999). Could interactions with homeobox proteins and FKBP52 permit nuclear receptors such as GR to appear at the cell surface, and even translocate between cells? If so, steroid signaling would take on an entirely new dimension.

## CONCLUSIONS AND OBSERVATIONS

The primary objective of this review has been to overview what is known of neurosteroids and their mechanism of action. We have been inextricably drawn towards the notion that intracellular and extracellular signaling may have a common origin, and steroids are likely to have intracellular targets operating at the level of sterol metabolism. But it would be a mistake to think that neurosteroids can only act in this way. Brain steroids can have acute actions, exemplified by the anxiogenic and proconvulsant effects of "natural" withdrawal of progesterone (Morau *et al.*, 1998). Testosterone elevations may contribute to elation following transient stress, while glucocorticoids have been postulated to play a role in

memory selection, facilitating the long-term storage of memory traces coincident with adrenal activation (Lathe, 2001). Other steroids whose levels vary according to time of day, such as DHEA, are likely to modulate mood, alertness and attention, while the onset, maintenance and termination of sleep in humans is likely to be governed, at least partly, by steroid signaling.

By way of conclusion, we have attempted to crystallize our thoughts into a shortlist of observations, some of which may stand the test of time, others of which may be less robust but we hope may point the way to new avenues of exploration. There can be no doubt that the field offers many new and exciting opportunities for future research.

- 1 The evidence argues against major levels of neurosteroid synthesis in brain; most brain steroids appear to derive from the circulation. At the same time, the brain is largely self-sufficient in cholesterol, and exploits a unique 24(S) hydroxylation and export pathway.
- 2 In contrast to local *de novo* steroid synthesis, local steroid transformations in brain are likely to be of importance. For instance, metabolism can regulate steroid access to receptors, exemplified by inactivation of corticosterone by 11B-HSD, and potentially could regulate the type of interaction (activation versus inhibition).
- 3 Local steroid metabolism may play a significant role, but the synthesis and metabolism of cholesterol and its oxysterol derivatives is of crucial importance to brain development and function. This is demonstrated by:
  - (a) the major brain effects of anxiolytics, anticonvulsants and neuroleptics that target intracellular sites regulating sterol transport and metabolism. In addition to more classic actions on neurotransmitter receptors (e.g., GABA<sub>A</sub> and dopamine receptors), benzodiazepines potently target the peripheral benzodiazepine receptor, PBR (sterol transport) while neuroleptics such as haloperidol target the sigma site and/or emapomil binding protein, EBP (involved in sterol synthesis);
  - (b) developmental and functional deficits of the brain due to abnormalities of cholesterol synthesis and metabolism, exemplified by Smith-Lemli-Opitz syndrome (cholesterol synthesis), Niemann-Pick disease type C (intracellular sterol transport), and cerebrotendinous xanthomatosis (cholesterol 27-hydroxylation).
- 4 Local intracellular sterol signaling includes:
  - (a) regulation of cholesterol synthesis and cell division;
  - (b) onset of apoptosis, and, potentially;
  - (c) membrane-dependent neurotransmitter release and subsequent receptor responses.
- 5 Systemic steroid signaling operates not only at classical nuclear "genomic" targets, but also at non-genomic sites including cell-surface neurotransmitter receptors (e.g., GABA<sub>A</sub>, NMDA and others) and intracellular sites including sigma/EBP (sterol synthesis) and PBR (sterol transport). From an evolutionary perspective, intracellular

- regulation of sterol synthesis and metabolism pre-dates systemic signaling; intracellular (and enzymatically-active) sites may be the earliest (and most important?) targets of steroid action (Stoka, 1999).
- 6 Local sterol signaling could potentially extend between cells by taking advantage of a reverse transport pathway from the interior to the cell surface, and uptake pathways to adjacent cells. This could contribute to developmental wiring in the nervous system, including synaptogenesis.
  - 7 Steroid signaling may also operate via "nuclear" receptors stationed at or near the cell surface. These may traffic to the cell surface via the same reverse transport system.
  - 8 There is extensive cross-talk between activity of neurotransmitter receptors at the cell surface (e.g., GABA<sub>A</sub>) and pathways operating within the cell (e.g., sigma, EBP, PBR). Both may be targets for (a) steroids and possibly sterols (b) the diazepam-binding inhibitor, DBI/endozapine. For instance, GABA-active agents can affect cholesterol synthesis. Cross-talk may also operate through second-messenger cascades and by coupling between cell membrane and intracellular (including mitochondrial) ion fluxes.
  - 9 Intracellular sterol metabolism, particularly in the mitochondrion, may decline with age. This could have consequences both at the systemic level (excess glucocorticoid production by the adrenal) and locally (neuronal loss). The abundant secretion of the steroid dehydroepiandrosterone (DHEA), in primates but not in rodents, could act to brake the age-related decline in sterol metabolism by operating at intracellular sterol sites.
  - 10 Finally, glimpses are emerging that selective metabolism of steroids and sterols may be localized within specialized cell regions, and possibly to active regions such as synapses. Potentially, this could permit fast steroid interconversions to govern brain activity at immediate (1sec-1min) time intervals.

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#### REFERENCES

- Ainsworth, L. and Nitchuk, W.M. (1975) Biotransformation of pregnenolone-7 $\alpha$ -<sup>3</sup>H, progesterone-7 $\alpha$ -<sup>3</sup>H and dehydroepiandrosterone-7 $\alpha$ -<sup>3</sup>H by porcine fetal and maternal adrenal homogenate preparations. *Steroids* **26**, 73–91.
- Ajilore, O.A. and Sapolsky, R.M. (1999) *In vivo* characterization of 11 $\beta$ -hydroxysteroid dehydrogenase in rat hippocampus using glucocorticoid neuroendangerment as an endpoint. *Neuroendocrinology* **69**, 138–144.
- Akwa, Y., Sananes, N., Gouezou, M., Robel, P., Baulieu, E.E. and Le Goascogne, C. (1993) Astrocytes and neurosteroids: metabolism of pregnenolone and dehydroepiandrosterone. Regulation by cell density. *J. Cell Biol.* **121**, 135–143.
- Akwa, Y., Young, J., Kabbadj, K., Sancho, M.J., Zucman, D., Vourc'h, C., Jung-Testas, I., Hu, Z.Y., Le Goascogne, C. and Jo, D.H. (1991) Neurosteroids: biosynthesis, metabolism and

- function of pregnenolone and dehydroepiandrosterone in the brain. *J. Steroid Biochem. Molec. Biol.* **40**, 71–81.
- Albiston, A.L., Obeyesekere, V.R., Smith, R.E. and Krozowski, Z.S. (1994) Cloning and tissue distribution of the human 11 $\beta$ -hydroxysteroid dehydrogenase type 2 enzyme. *Mol. Cell. Endocrinol.* **105**, R11–7.
- Alho, H., Fremeau, Jr. R.T., Tiedge, H., Wilcox, J., Bovolín, P., Brosius, J., Roberts, J.L. and Costa, E. (1988) Diazepam binding inhibitor gene expression: location in brain and peripheral tissues of rat. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7018–7022.
- Allera, A. and Wildt, L. (1992a) Glucocorticoid-recognizing and -effector sites in rat liver plasma membrane. Kinetics of corticosterone uptake by isolated membrane vesicles: I. Binding and transport. *J. Steroid. Biochem. Molec. Biol.* **42**, 737–756.
- Allera, A. and Wildt, L. (1992b) Glucocorticoid-recognizing and -effector sites in rat liver plasma membrane. Kinetics of corticosterone uptake by isolated membrane vesicles: II. Comparative influx and efflux. *J. Steroid. Biochem. Molec. Biol.* **42**, 757–771.
- Altman, J. and Das, G.D.D. (1965) Post-natal origin of microneurons in the rat brain. *Nature* **207**, 953–956.
- Anderson, A.J., Stephan, M.J., Walker, W.O. and Kelley, R.I. (1998) Variant RSH/Smith-Lemli-Opitz syndrome with atypical sterol metabolism. *Am. J. Med. Genet.* **78**, 413–418.
- Antoni, F.A., Palkovits, M., Simpson, J., Smith, S.M., Leitch, A.L., Rosie, R., Fink, G. and Paterson, J.M. (1998) Ca<sup>2+</sup>/calcineurin-inhibited adenylyl cyclase, highly abundant in forebrain regions, is important for learning and memory. *J. Neurosci.* **18**, 9650–9661.
- Araneo, B. and Daynes, R. (1995) Dehydroepiandrosterone functions as more than an antiglucocorticoid in preserving immunocompetence after thermal injury. *Endocrinology* **136**, 393–401.
- Asai, A., Qiu, J., Narita, Y., Chi, S., Saito, N., Shinoura, N., Hamada, H., Kuchino, Y. and Kirino, T. (1999) High level calcineurin activity predisposes neuronal cells to apoptosis. *J. Biol. Chem.* **274**, 34450–34458.
- Aso, T. (1976) A radioimmunoassay method for simultaneous determination of pregnenolone, pregnenolone sulfate, dehydroepiandrosterone and dehydroepiandrosterone sulfate in human plasma. *Nippon Naibunpi Gakkai Zasshi* **52**, 1008–1019.
- Asthana, S., Craft, S., Baker, L.D., Raskind, M.A., Birnbaum, R.S., Lofgreen, C.P., Veith, R.C. and Plymate, S.R. (1999) Cognitive and neuroendocrine response to transdermal estrogen in postmenopausal women with Alzheimer's disease: results of a placebo-controlled, double-blind, pilot study. *Psychoneuroendocrinology* **24**, 657–677.
- Babcock-Atkinson, E., Norenberg, L.O., Norenberg, M.D. and Neary, J.T. (1989) Diazepam inhibits calcium, calmodulin-dependent protein kinase in primary astrocyte cultures. *Brain Res.* **484**, 399–403.
- Baker, M.E. (1997) Steroid receptor phylogeny and vertebrate origins. *Mol. Cell. Endocrinol.* **135**, 101–107.
- Balthazart, I. and Ball, G.F. (2000) Fast regulation of steroid biosynthesis: a further piece in the neurosteroid puzzle. *Trends Neurosci.* **23**, 57–58.
- Barbaccia, M.L., Berkovich, A., Guarneri, P. and Slobodyansky, E. (1990) DBI (diazepam binding inhibitor): the precursor of a family of endogenous modulators of GABA<sub>A</sub> receptor function. History, perspectives, and clinical implications. *Neurochem. Res.* **15**, 161–168.
- Baulieu, E.E. (1998) Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology* **23**, 963–987.
- Baulieu, E.E., Thomas, G., Legrain, S., Lahlou, N., Roger, M., Debuire, B., Faucounau, V., Girard, L., Hervy, M.P., Latour, F., *et al.* (2000) Dehydroepiandrosterone (DHEA), DHEA

- sulfate, and aging: contribution of the DHEAge Study to a sociobiomedical issue. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4279–4284.
- Behl, C., Rupprecht, R., Skutella, T. and Holsboer, F. (1995) Haloperidol-induced cell death—mechanism and protection with vitamin E *in vitro*. *Neuroreport* **7**, 360–364.
- Belelli, I., Pistis, I., Peters, J.A. and Lambert, J.J. (1999) General anaesthetic action at transmitter-gated inhibitory amino acid receptors. *Trends Pharmacol. Sci.* **20**, 496–502.
- Belloni, E., Muenke, M., Roessler, E., Traverse, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H.F., Donis-Keller, H., Helms, C., Hing, A.V., Heng, H.H., Koop, B., Martindale, D., Rommens, J.M., Tsui, L.C. and Scherer, S.W. (1996) Identification of Sonic hedgehog as a candidate gene responsible for holoprosencephaly. *Nat. Genet.* **14**, 353–356.
- Bergeron, R., de Montigny, C. and Debonnel, G. (1996) Potentiation of neuronal NMDA response induced by dehydroepiandrosterone and its suppression by progesterone: effects mediated via sigma receptors. *J. Neurosci.* **16**, 1193–1202.
- Beri, R., Kumar, N., Savage, T., Benalcazar, L. and Sundaram, K. (1998) Estrogenic and progestational activity of 7 $\alpha$ -methyl-19-nortestosterone, a synthetic androgen. *J. Steroid. Biochem. Molec. Biol.* **67**, 275–283.
- Beriozov, A.T., Ivanov, A.S., Ivkov, V.G., Obratsov, V.V., Khalilov, E.M. and Archakov, A.I. (1990) Cholesterol oxidation on fluorocarbon emulsion surface leads to the formation of 7-peroxy-cholesterol. *FEBS Lett.* **266**, 72–74.
- Bertics, S.J., Bertics, P.J., Clarke, J.L. and Karavolas, H.J. (1987) Distribution and ovarian control of progesterin-metabolizing enzymes in various rat hypothalamic regions. *J. Steroid. Biochem.* **26**, 321–328.
- Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., Ohlsson, R., Postlind, H., Blomquist, P. and Berkenstam, A. (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12208–12213.
- Besman, M.J., Yanagibashi, K., Lee, T.D., Kawamura, M., Hall, P.F. and Shively, J.E. (1989) Identification of des-(Gly-Ile)-endozepine as an effector of corticotropin-dependent adrenal steroidogenesis: stimulation of cholesterol delivery is mediated by the peripheral benzodiazepine receptor. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4897–4901.
- Beyer, C. (1999) Estrogen and the developing mammalian brain. *Anat. Embryol. (Berl.)* **199**, 379–390.
- Bhagwat, S.V., Biswas, G., Anandatheerthavarada, H.K., Addya, S., Pandak, W., Avadhani, N.G. and Mullick, J. (1997) Dual targeting property of the N-terminal signal sequence of P4501A1. Targeting of heterologous proteins to endoplasmic reticulum and mitochondria targeting of NH<sub>2</sub>-terminal-processed microsomal protein to mitochondria: a novel pathway for the biogenesis of hepatic mitochondrial P450MT2. *J. Cell Biol.* **139**, 589–599.
- Björkhem, I. (1994) Inborn errors of metabolism with consequences for bile acid biosynthesis: a minireview. *Scand. J. Gastroenterol.* **204S**, 68–72.
- Björkhem, I., Diczfalusy, U. and Lutjohann, D. (1999) Removal of cholesterol from extrahepatic sources by oxidative mechanisms. *Curr. Opin. Lipidol.* **10**, 161–165.
- Björkhem, I., Lutjohann, D., Breuer, O., Sakinis, A. and Wennmalm, A. (1997) Importance of a novel oxidative mechanism for elimination of brain cholesterol: turnover of cholesterol and 24(S)-hydroxycholesterol in rat brain as measured with 18O<sub>2</sub> techniques *in vivo* and *in vitro*. *J. Biol. Chem.* **272**, 30178–30184.
- Björkhem, I., Lutjohann, D., Diczfalusy, U., Stahle, L., Ahlborg, G. and Wahren, J. (1998) Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation. *J. Lipid Res.* **39**, 1594–1600.



- Blaschke, A.J., Staley, K. and Chun, J. (1996) Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. *Development* **122**, 1165–1174.
- Blaschke, A.J., Weiner, J.A. and Chun, J. (1998) Programmed cell death is a universal feature of embryonic and postnatal neuroproliferative regions throughout the central nervous system. *J. Comp. Neurol.* **396**, 39–50.
- Blauer, K.L., Poth, M., Rogers, W.M. and Bernton, E.W. (1991) Dehydroepiandrosterone antagonizes the suppressive effects of dexamethasone on lymphocyte proliferation. *Endocrinology* **129**, 3174–3179.
- Blumberg, B., Sabbagh, Jr. W., Juguilon, H., Bolado, Jr. J., van Meter, C.M., Ong, E.S. and Evans, R.M. (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **12**, 3195–3205.
- Bono, F., Lamarche, I., Prabonnaud, V., Le Fur, G. and Herbert, J.M. (1999) Peripheral benzodiazepine receptor agonists exhibit potent antiapoptotic activities. *Biochem. Biophys. Res. Commun.* **265**, 457–461.
- Bouchard, P. and Quirion, R. (1997) [<sup>3</sup>H]1,3-di(2-tolyl) guanidine and [<sup>3</sup>H](+)-pentazocine binding sites in the rat brain: autoradiographic visualization of the putative sigma1 and sigma2 receptor subtypes. *Neuroscience* **76**, 467–477.
- Bourre, J.M., Clement, M., Gerard, D., Legrand, R. and Chaudiere, J. (1990) Precursors for cholesterol synthesis (7-dehydrocholesterol, 7-dehydrosesmosterol, and desmosterol): cholesterol/7-dehydrocholesterol ratio as an index of development and aging in PNS but not in CNS. *J. Neurochem.* **54**, 1196–1199.
- Brand, C., Cherradi, N., Defaye, G., Chinn, A., Chambaz, E.M., Feige, J.J. and Bailly, S. (1998) Transforming growth factor beta1 decreases cholesterol supply to mitochondria via repression of steroidogenic acute regulatory protein expression. *J. Biol. Chem.* **273**, 6410–6416.
- Brent, P.J., Pang, G., Little, G., Dosen, P.J. and Van Helden, D.F. (1996) The sigma receptor ligand, reduced haloperidol, induces apoptosis and increases in trace llular-free calcium levels [ $\text{Ca}^{2+}$ ]<sub>i</sub> in colon and mammary adenocarcinoma cells. *Biochem. Biophys. Res. Commun.* **219**, 219–226.
- Brinton, R.D., Proffitt, P., Tran, J. and Luu, R. (1997) Equilin, a principal component of the estrogen replacement therapy premarin, increases the growth of cortical neurons via an NMDA receptor-dependent mechanism. *Exp. Neurol.* **147**, 211–220.
- Brown, M.S. and Goldstein, J.L. (1997) The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**, 331–340.
- Brown, R.W., Diaz, R., Robson, A.C., Kotelevtsev, Y.V., Mullins, J.J., Kaufman, M.H. and Seckl, J.R. (1996) The ontogeny of 11 $\beta$ -hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology* **137**, 794–797.
- Brown, S.J., Hilgenfeld, R.B. and Denell, R.E. (1994) The beetle *Tribolium castaneum* has a fushi tarazu homolog expressed in stripes during segmentation. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12922–12926.
- Bullock, L.P., Feil, P.D., Gupta, C., Demers, L.M. and Bardin, C.W. (1978) Androgenic effects of the antiprogesterone RMI 12,936. *J. Reprod. Fertil.* **52**, 365–371.
- Cali, J.J., Hsieh, C.L., Francke, U. and Russell, D.W. (1991) Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. *J. Biol. Chem.* **266**, 7779–7783.
- Cali, J.J. and Russell, D.W. (1991) Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidation reaction in bile acid biosynthesis. *J. Biol. Chem.* **266**, 7774–7778.

- Calleja, C., Pascussi, J.M., Mani, J.C., Maurel, P. and Vilarem, M.J. (1998) The antibiotic rifampicin is a nonsteroidal ligand and activator of the human glucocorticoid receptor. *Nature Med.* **4**, 92–96.
- Cardounel, A., Regelson, W. and Kalimi, M. (1999) Dehydroepiandrosterone protects hippocampal neurons against neurotoxin-induced cell death: mechanism of action. Dehydroepiandrosterone protects hippocampal neurons against neurotoxin-induced cell death: mechanism of action. *Proc. Soc. Exp. Biol. Med.* **222**, 145–149.
- Carey, M.P., Aniszewski, C.A. and Fry, J.P. (1994) Metabolism of progesterone in mouse brain. *J. Steroid. Biochem. Molec. Biol.* **50**, 213–217.
- Caron-Leslie, L.M., Schwartzman, R.A., Gaido, M.L., Compton, M.M. and Cidlowski, J.A. (1991) Identification and characterization of glucocorticoid-regulated nuclease(s) in lymphoid cells undergoing apoptosis. *J. Steroid. Biochem. Molec. Biol.* **40**, 661–671.
- Cascio, C., Prasad, V.V., Lin, Y.Y., Lieberman, S. and Papadopoulos, V. (1998) Detection of P450c17-independent pathways for dehydroepiandrosterone (DHEA) biosynthesis in brain glial tumor cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2862–2867.
- Chang, J.Y. and Liu, L.Z. (1998) Neurotoxicity of cholesterol oxides on cultured cerebellar granule cells. *Neurochem. Int.* **32**, 317–323.
- Cheney, D.L., Uzunov, D., Costa, E. and Guidotti, A. (1995) Gas chromatographic-mass fragmentographic quantitation of 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnan-20-one (allopregnanolone) and its precursors in blood and brain of adrenalectomized and castrated rats. *J. Neurosci.* **15**, 4641–4650.
- Cheng, K.C., Lee, J., Khanna, M. and Qin, K.N. (1994) Distribution and ontogeny of 3-hydroxysteroid dehydrogenase in the rat brain. *J. Steroid. Biochem. Molec. Biol.* **50**, 85–89.
- Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H. and Beachy, P.A. (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407–413.
- Chisolm, G.M., Ma, G., Irwin, K.C., Martin, L.L., Gunderson, K.G., Linberg, L.F., Morel, D.W. and DiCorleto, P.E. (1994) 7 $\beta$ -hydroperoxycholest-5-en-3 $\beta$ -ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low density lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11452–11456.
- Chmielewski, V., Drupt, F. and Morfin, R. (2000) Dexamethasone-induced apoptosis of mouse thymocytes: prevention by native 7 $\alpha$ -hydroxysteroids. *Immunol. Cell Biol.* **78**, 238–246.
- Choi, I., Troyer, D.L., Cornwell, D.L., Kirby-Dobbels, K.R., Collante, W.R. and Simmen, F.A. (1997) Closely related genes encode developmental and tissue isoforms of porcine cytochrome P450 aromatase. *DNA Cell Biol.* **16**, 769–777.
- Christ, M., Luu, B., Mejia, J.E., Moosbrugger, I. and Bischoff, P. (1993) Apoptosis induced by oxysterols in murine lymphoma cells and in normal thymocytes. *Immunology* **78**, 455–460.
- Church, J. and Fletcher, E.J. (1995) Blockade by sigma site ligands of high voltage-activated Ca<sup>2+</sup> channels in rat and mouse cultured hippocampal pyramidal neurons. *Br. J. Pharmacol.* **116**, 2801–2810.
- Cifone, M.G., Migliorati, G., Parroni, R., Marchetti, C., Millimaggi, D., Santoni, A. and Riccardi, C. (1999) Dexamethasone-induced thymocyte apoptosis: apoptotic signal involves the sequential activation of phosphoinositide-specific phospholipase C, acidic sphingomyelinase, and caspases. *Blood* **93**, 2282–2296.
- Clarke, C.H., Norfleet, A.M., Clarke, M.S., Watson, C.S., Cunningham, K.A. and Thomas, M.L. (2000) Perimembrane localization of the estrogen receptor a protein in neuronal processes of cultured hippocampal neurons. *Neuroendocrinology* **71**, 34–42.

- Clarke, T.R., Bain, P.A., Greco, T.L. and Payne, A.H. (1993) A novel mouse kidney 3 $\beta$ -hydroxy steroid dehydrogenase complementary DNA encodes a 3-ketosteroid reductase instead of a 3 $\beta$ -hydroxysteroid dehydrogenase/delta 5-delta 4-isomerase. *Mol. Endocrinol.* **7**, 1569–1578.
- Clemens, L.G. and Gladue, B.A. (1978) Feminine sexual behavior in rats enhanced by prenatal inhibition of androgen aromatization. *Horm. Behav.* **11**, 190–201.
- Compagnone, N.A., Bulfone, A., Rubenstein, J.L. and Mellon, S.H. (1995a) Steroidogenic enzyme P450c17 is expressed in the embryonic central nervous system. *Endocrinology* **136**, 5212–5223.
- Compagnone, N.A., Bulfone, A., Rubenstein, J.L. and Mellon, S.H. (1995b) Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis. *Endocrinology* **136**, 2689–2696.
- Cooke, B., Hegstrom, C.D., Villeneuve, L.S. and Breedlove, S.M. (1998) Sexual differentiation of the vertebrate brain: principles and mechanisms. *Front. Neuroendocrinol.* **19**, 323–362.
- Cooper, M.K., Porter, J.A., Young, K.E. and Beachy, P.A. (1998) Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280**, 1603–1607.
- Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**, 921–923.
- Corpechot, C., Leclerc, P., Baulieu, E.E. and Brazeau, P. (1985) Neurosteroids: regulatory mechanisms in male rat brain during heterosexual exposure. *Steroids* **45**, 229–234.
- Corpechot, C., Robel, P., Axelsson, M., Sjoval, J. and Baulieu, E.E. (1981) Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4704–4707.
- Corpechot, C., Synguelakis, M., Talha, S., Axelsson, M., Sjoval, J., Vihko, R., Baulieu, E.E. and Robel, P. (1983) Pregnenolone and its sulfate ester in the rat brain. *Brain Res.* **270**, 119–125.
- Costa, E. and Guidotti, A. (1991) Diazepam binding inhibitor (DBI): a peptide with multiple biological actions. *Life Sci.* **49**, 325–344.
- Couch, R.A., Skinner, S.J., Tobler, C.J. and Doouss, T.W. (1975) The *in vitro* synthesis of 7-hydroxy dehydroepiandrosterone by human mammary tissues. *Steroids* **26**, 1–15.
- Couture, S. and Debonnel, G. (1998) Modulation of the neuronal response to N-methyl-D-aspartate by selective sigma2 ligands. *Synapse* **29**, 62–71.
- Crompton, M., Virji, S., Doyle, V., Johnson, N. and Ward, J.M. (1999) The mitochondrial permeability transition pore. *Biochem. Soc. Symp.* **66**, 167–179.
- Crossin, K.L., Tai, M.H., Krushel, L.A., Mauro, V.P. and Edelman, G.M. (1997) Glucocorticoid receptor pathways are involved in the inhibition of astrocyte proliferation. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2687–2692.
- Crossley, K.J., Nicol, M.B., Hirst, J.J., Walker, D.W. and Thorburn, G.D. (1997) Suppression of arousal by progesterone in fetal sheep. *Reprod. Fertil. Dev.* **9**, 767–773.
- Culty, M., Li, H., Boujrad, N., Amri, H., Vidic, B., Bernassau, J.M., Reversal, J.L. and Papadopoulos, V. (1999) *In vitro* studies on the role of the peripheral-type benzodiazepine receptor in steroidogenesis. *J. Steroid. Biochem. Molec. Biol.* **69**, 123–130.
- Dahlback-Sjoberg, H., Björkhem, I. and Princen, H.M. (1993) Selective inhibition of mitochondrial 27-hydroxylation of bile acid intermediates and 25-hydroxylation of vitamin D<sub>3</sub> by cyclosporin A. *Biochem. J.* **293**, 203–206.
- Davis, E.G., Popper, P. and Gorski, R.A. (1996) The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area. *Brain Res.* **734**, 10–18.
- Dawson, T.M., Steiner, J.P., Dawson, V.L., Dinerman, J.L., Uhl, G.R. and Snyder, S.H. (1993) Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9808–9812.

- Daynes, R.A., Dudley, D.J. and Araneo, B.A. (1990) Regulation of murine lymphokine production *in vivo*. II. Dehydroepiandrosterone is a natural enhancer of interleukin 2 synthesis by helper T cells. *Eur. J. Immunol.* **20**, 793–802.
- De Kloet, E.R., Vreugdenhil, E., Oitzl, M.S. and Joels, M. (1998) Brain corticosteroid receptor balance in health and disease. *Endocr. Rev.* **19**, 269–301.
- Debonnel, G., Bergeron, R. and de Montigny, C. (1996) Potentiation by dehydroepiandrosterone of the neuronal response to N-methyl-D-aspartate in the CA3 region of the rat dorsal hippocampus: an effect mediated via sigma receptors. *J. Endocrinol.* **150**, S33–42.
- DeFriend, D.J., Howell, A., Nicholson, R.I., Anderson, E., Dowsett, M., Mansel, R.E., Blarney, R.W., Bundred, N.J., Robertson, J.F. and Saunders, C. (1994) Investigation of a new pure antiestrogen (ICI 182780) in women with primary breast cancer. *Cancer Res.* **54**, 408–414.
- Dehouck, B., Fenart, L., Dehouck, M.P., Pierce, A., Torpier, G. and Cecchelli, R. (1997) A new function for the LDL receptor: transcytosis of LDL across the blood-brain barrier. *J. Cell Biol.* **138**, 877–889.
- Derocq, J.M., Bourrie, B., Segui, M., Le Fur, G. and Casellas, P. (1995) *In vivo* inhibition of endotoxin-induced pro-inflammatory cytokines production by the sigma ligand SR 31747. *J. Pharmacol. Exp. Ther.* **272**, 224–230.
- Derossi, D., Calvet, S., Trembleau, A., Brunissen, A., Chassaing, G. and Prochiantz, A. (1996) Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* **271**, 18188–18193.
- Diamond, D.M., Fleshner, M. and Rose, G.M. (1999) The enhancement of hippocampal primed burst potentiation by dehydroepiandrosterone sulfate (DHEAS) is blocked by psychological stress. *Stress* **3**, 107–121.
- Diaz, R., Brown, R.W. and Seckl, J.R. (1998) Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11 $\beta$ -hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. *J. Neurosci.* **18**, 2570–2580.
- Doostzadeh, J., Cotillon, A.C., Benalycherif, A. and Morfin, R. (1998a) Inhibition studies of dehydroepiandrosterone 7 $\alpha$ - and 7 $\beta$ -hydroxylation in mouse liver microsomes. *Steroids* **63**, 608–614.
- Doostzadeh, J., Cotillon, A.C. and Morfin, R. (1997) Dehydroepiandrosterone 7 $\alpha$ - and 7 $\beta$ -hydroxylation in mouse brain microsomes. Effects of cytochrome P450 inhibitors and structure-specific inhibition by steroid hormones. *J. Neuroendocrinol.* **9**, 923–928.
- Doostzadeh, J., Cotillon, A.C. and Morfin, R. (1998b) Hydroxylation of pregnenolone at the 7 $\alpha$ - and 7 $\beta$ -positions by mouse liver microsomes. Effects of cytochrome P450 inhibitors and structure-specific inhibition by steroid hormones. *Steroids* **63**, 383–392.
- Doostzadeh, J. and Morfin, R. (1997) Effects of cytochrome P450 inhibitors and of steroid hormones on the formation of 7-hydroxylated metabolites of pregnenolone in mouse brain microsomes. *J. Endocrinol.* **155**, 343–350.
- Duhamel-Clerin, E., Villarroja, H., Mehtali, M., Lapie, P., Besnard, F., Gumpel, M. and Lachapelle, F. (1994) Cellular expression of an HMGCR promoter-CAT fusion gene in transgenic mouse brain: evidence for a developmental regulation in oligodendrocytes. *Glia* **11**, 35–46.
- Dunn, J.F., Nisula, B.C. and Rodbard, D. (1981) Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* **53**, 58–68.
- Eaton, M.J., Lookingland, K.J. and Moore, K.E. (1996) The sigma ligand rimcazone activates noradrenergic neurons projecting to the paraventricular nucleus and increases corticosterone secretion in rats. *Brain Res.* **733**, 162–166.

- Edwards, C.R., Stewart, P.M., Burt, D., Brett, L., McIntyre, M.A., Sutanto, W.S., De Kloet, E.R. and Monder, C. (1988) Localisation of 11 $\beta$ -hydroxysteroid dehydrogenase-tissue specific protector of the mineralocorticoid receptor. *Lancet* **2**, 986–989.
- Edwards, O.M., Courtenay-Evans, R.J., Galley, J.M., Hunter, J. and Tait, A.D. (1974) Changes in cortisol metabolism following rifampicin therapy. *Lancet* **2**, 548–551.
- Erdmann, B., Gerst, H., Lippoldt, A., Bulow, H., Ganten, D., Fuxe, K. and Bernhardt, R. (1996) Expression of cytochrome P45011B1 mRNA in the brain of normal and hypertensive transgenic rats. *Brain Res.* **733**, 73–82.
- Farese, Jr. R.V., Ruland, S.L., Flynn, L.M., Stokowski, R.P. and Young, S.G. (1995) Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1774–1778.
- Fawcett, J., Busch, K.A., Jacobs, D., Kravitz, H.M. and Fogg, L. (1997) Suicide: a four-pathway clinical-biochemical model. *Ann. NY Acad. Sci.* **836**, 288–301.
- Ferrand-Drake, M., Friberg, H. and Wieloch, T. (1999) Mitochondrial permeability transition induced DNA-fragmentation in the rat hippocampus following hypoglycemia. *Neuroscience* **90**, 1325–1338.
- Ferrarese, C., Marzorati, C., Perego, M., Bianchi, G., Cavarretta, R., Pierpaoli, C., Moretti, G. and Frattola, L. (1995) Effect of anticonvulsant drugs on peripheral benzodiazepine receptors of human lymphocytes. *Neuropharmacology* **34**, 427–431.
- Fisher, C.R., Graves, K.H., Parlow, A.F. and Simpson, E.R. (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the CYP19 gene. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6965–6970.
- Fishman, J. and Norton, B. (1975) Catechol estrogen formation in the central nervous system of the rat. *Endocrinology* **96**, 1054–1058.
- Fitzky, B.U., Glossmann, H., Utermann, G. and Moebius, F.F. (1999) Molecular genetics of the Smith-Lemli-Opitz syndrome and postsqualene sterol metabolism. *Curr. Opin. Lipidol.* **10**, 123–131.
- Fleshner, M., Pugh, C.R., Tremblay, D. and Rudy, J.W. (1997) DHEA-S selectively impairs contextual-fear conditioning: support for the antigluccorticoid hypothesis. *Behav. Neurosci.* **111**, 512–517.
- Flood, J.F., Morley, J.E. and Roberts, E. (1992) Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1567–1571.
- Flood, J.F. and Roberts, E. (1988) Dehydroepiandrosterone sulfate improves memory in aging mice. *Brain Res.* **448**, 178–181.
- Forman, B.M., Tzamelis, I., Choi, H.S., Chen, J., Simha, D., Seol, W., Evans, R.M. and Moore, D.D. (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR- $\beta$ . *Nature* **395**, 612–615.
- Funder, J.W., Pearce, P.T., Smith, R. and Smith, A.I. (1988) Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242**, 583–585.
- Furukawa, A., Miyatake, A., Ohnishi, T. and Ichikawa, Y. (1998) Steroidogenic acute regulatory protein (StAR) transcripts constitutively expressed in the adult rat central nervous system: colocalization of StAR, cytochrome P-450SCC (CYP XIA1), and 3 $\beta$ -hydroxysteroid dehydrogenase in the rat brain. *J. Neurochem.* **71**, 2231–2238.
- Galiegue, S., Jbilo, O., Combes, T., Bribes, E., Carayon, P., Le Fur, G. and Casellas, P. (1999) Cloning and characterization of PRAX-1: a new protein that specifically interacts with the peripheral benzodiazepine receptor. *J. Biol. Chem.* **274**, 2938–2952.

- Gametchu, B., Chen, F., Sackey, F., Powell, C. and Watson, C.S. (1999) Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models. *Steroids* **64**, 107–119.
- Gemzik, B., Green, J. and Parkinson, A. (1992a) Hydroxylation of 5 $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol by rat prostate microsomes: effects of antibodies and chemical inhibitors of cytochrome P450 enzymes. *Arch. Biochem. Biophys.* **296**, 355–365.
- Gemzik, B., Jacob, S., Jennings, S., Veltman, J. and Parkinson, A. (1992b) Species differences in 5 $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol hydroxylation by rat, monkey, and human prostate microsomes. *Arch. Biochem. Biophys.* **296**, 374–383.
- Gemzik, B. and Parkinson, A. (1992) Hydroxylation of 5 $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol by rat prostate microsomes: potent inhibition by imidazole-type antimycotic drugs and lack of inhibition by steroid 5 $\alpha$ -reductase inhibitors. *Arch. Biochem. Biophys.* **296**, 366–373.
- Gijtenbeek, J.M., van den Bent, M.J. and Vecht, C.J. (1999) Cyclosporine neurotoxicity: a review. *J. Neurol.* **246**, 339–346.
- Gold, B.C., Densmore, V., Shou, W., Matzuk, M.M. and Gordon, H.S. (1999) Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. *J. Pharmacol. Exp. Ther.* **289**, 1202–1210.
- Gomez-Sanchez, C.E., Zhou, M.Y., Cozza, E.N., Morita, H., Eddleman, F.C. and Gomez-Sanchez, E.P. (1996) Corticosteroid synthesis in the central nervous system. *Endocr. Res.* **22**, 463–470.
- Gomez-Sanchez, C.E., Zhou, M.Y., Cozza, E.N., Morita, H., Foeking, M.F. and Gomez-Sanchez, E.P. (1997) Aldosterone biosynthesis in the rat brain. *Endocrinology* **138**, 3369–3373.
- Gomez-Sanchez, E.P. (1986) Intracerebroventricular infusion of aldosterone induces hypertension in rats. *Endocrinology* **118**, 819–823.
- Gomez-Sanchez, E.P. and Gomez-Sanchez, C.E. (1992) Central hypertensinogenic effects of glycyrrhizic acid and carbenoxolone. *Am. J. Physiol.* **263**, E125–E130.
- Goodwin, B., Hodgson, E. and Liddle, C. (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol. Pharmacol.* **56**, 1329–1339.
- Gordon, I., Grauer, E., Genis, I., Sehayek, E. and Michaelson, D.M. (1995) Memory deficits and cholinergic impairments in apolipoprotein E-deficient mice. *Neurosci. Lett.* **199**, 1–4.
- Gould, E., Cameron, H.A., Daniels, D.C., Woolley, C.S. and McEwen, B.S. (1992) Adrenal hormones suppress cell division in the adult rat dentate gyrus. *J. Neurosci.* **12**, 3642–3650.
- Gould, E., Woolley, C.S. and McEwen, B.S. (1990) Short-term glucocorticoid manipulations affect neuronal morphology and survival in the adult dentate gyrus. *Neuroscience* **37**, 367–375.
- Gray, P.W., Glaister, D., Seeburg, P.H., Guidotti, A. and Costa, E. (1986) Cloning and expression of cDNA for human diazepam binding inhibitor, a natural ligand of an allosteric regulatory site of the gamma-aminobutyric acid type A receptor. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7547–7551.
- Gu, Q., Korach, K.S. and Moss, R.L. (1999) Rapid action of 17 $\beta$ -estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. *Endocrinology* **140**, 660–666.
- Guarneri, P., Guarneri, R., Cascio, C., Pavasant, P., Piccoli, F. and Papadopoulos, V. (1994) Neurosteroidogenesis in rat retinas. *J. Neurochem.* **63**, 86–96.
- Guarneri, P., Guarneri, R., Cascio, C., Piccoli, F. and Papadopoulos, V. (1995) gamma-Aminobutyric acid type A/benzodiazepine receptors regulate rat retina neurosteroidogenesis. *Brain Res.* **683**, 65–72.

- Guazzo, E.P., Kirkpatrick, P.J., Goodyer, I.M., Shiers, H.M. and Herbert, J. (1996) Cortisol, dehydroepiandrosterone (DHEA), and DHEA sulfate in the cerebrospinal fluid of man: relation to blood levels and the effects of age. *J. Clin. Endocrinol. Metab.* **81**, 3951–3960.
- Gudelsky, G.A. and Nash, J.F. (1992) Neuroendocrinological and neurochemical effects of sigma ligands. *Neuropharmacology* **31**, 157–162.
- Guennoun, R., Fiddes, R.J., Gouezou, M., Lombes, M. and Baulieu, E.E. (1995) A key enzyme in the biosynthesis of neurosteroids, 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$ -isomerase (3 $\beta$ -HSD), is expressed in rat brain. *Brain Res. Molec. Brain Res.* **30**, 287–300.
- Guennoun, R., Schumacher, M., Robert, F., Delespierre, B., Gouezou, M., Eychemme, B., Akwa, Y., Robel, P. and Baulieu, E.E. (1997) Neurosteroids: expression of functional 3 $\beta$ -hydroxy steroid dehydrogenase by rat sensory neurons and Schwann cells. *Eur. J. Neurosci.* **9**, 2236–2247.
- Guichet, A., Copeland, J.W., Erdelyi, M., Hlousek, D., Zavorsky, P., Ho, J., Brown, S., Percival-Smith, A., Krause, H.M. and Ephrussi, A. (1997) The nuclear receptor homologue Ftz-F1 and the homeodomain protein Ftz are mutually dependent cofactors. *Nature* **385**, 548–552.
- Gunther, U., Benson, J., Benke, D., Fritschy, J.M., Reyes, G., Knoflach, F., Crestani, F., Aguzzi, A., Arigoni, M. and Lang, Y. (1995) Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7749–7753.
- Gustafsson, J.A. (1999) Seeking ligands for lonely orphan receptors. *Science* **284**, 1285–1286.
- Hackenberg, R., Turgetto, I., Filmer, A. and Schulz, K.D. (1993) Estrogen and androgen receptor mediated stimulation and inhibition of proliferation by androst-5-ene-3 $\beta$ , 17 $\beta$ -diol in human mammary cancer cells. *J. Steroid. Biochem. Molec. Biol.* **46**, 597–603.
- Hanner, M., Moebius, F.F., Flandorfer, A., Knaus, H.G., Striessnig, J., Kempner, E. and Glossmann, H. (1996) Purification, molecular cloning, and expression of the mammalian sigma 1-binding site. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8072–8077.
- Harris, H.J., Kotelevtsev, Y., Mullins, J.J., Seckl, J.R. and Holmes, M.C. (2000). 11 $\beta$ -hydroxysteroid dehydrogenase type 1 null mice have altered hypothalamic-pituitary-adrenal axis activity: a novel control of glucocorticoid feedback. *Endocrinology* **141**, in press.
- Harrison, N.L. and Simmonds, M.A. (1984) Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res.* **323**, 287–292.
- Haskell, S.G., Richardson, E.D. and Horwitz, R.I. (1997) The effect of estrogen replacement therapy on cognitive function in women: a critical review of the literature. *J. Clin. Epidemiol.* **50**, 1249–1264.
- Hauben, M. (1996) Cyclosporine neurotoxicity. *Pharmacotherapy* **16**, 576–583.
- Hayashi, T., Maurice, T. and Su, T.P. (2000) Ca(2+) signaling via sigma(1)-receptors: novel regulatory mechanism affecting intracellular Ca(2+) concentration. *J. Pharmacol. Exp.* **293**, 788–798.
- Hermans-Borgmeyer, I., Hampe, W., Schinke, B., Methner, A., Nykjaer, A., Susens, U., Fenger, U., Herbarth, B. and Schaller, H.C. (1998) Unique expression pattern of a novel mosaic receptor in the developing cerebral cortex. *Mech. Dev.* **70**, 65–76.
- Hinson, J.P. and Raven, P.W. (1999) DHEA deficiency syndrome: a new term for old age? *J. Endocrinol.* **163**, 1–5.
- Hirsch, T., Decaudin, D., Susin, S.A., Marchetti, P., Larochette, N., Resche-Rigon, M. and Kroemer, G. (1998) PK11195, a ligand of the mitochondrial benzodiazepine receptor, facilitates the induction of apoptosis and reverses Bcl-2-mediated cytoprotection. *Exp. Cell Res.* **241**, 426–434.

- Hofmann, T.G., Hehner, S.P., Bacher, S., Droge, W. and Schmitz, M.L. (1998) Various glucocorticoids differ in their ability to induce gene expression, apoptosis and to repress NF-kappaB-dependent transcription. *FEBS Lett.* **441**, 441–446.
- Holick, M.F. (1995) Noncalcemic actions of 1,25-dihydroxyvitamin D<sub>3</sub> and clinical applications. *Bone* **17**, 107S–111S.
- Homanics, G.E., Maeda, N., Traber, M.G., Kayden, H.J., Dehart, D.B. and Sulik, K.K. (1995) Exencephaly and hydrocephaly in mice with targeted modification of the apolipoprotein B (Apo B) gene. *Teratology* **51**, 1–10.
- Honda, S., Harada, N., Ito, S., Takagi, Y. and Maeda, S. (1998) Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the CYP19 gene. *Biochem. Biophys. Res. Commun.* **252**, 445–449.
- Honda, S., Harada, N. and Takagi, Y. (1994) Novel exon 1 of the aromatase gene specific for aromatase transcripts in human brain. *Biochem. Biophys. Res. Commun.* **198**, 1153–1160.
- Honjo, H., Ogino, Y., Naitoh, K., Urabe, M., Kitawaki, J., Yasuda, J., Yamamoto, T., Ishihara, S., Okada, H. and Yonezawa, T. (1989) *In vivo* effects by estrone sulfate on the central nervous system-senile dementia (Alzheimer's type). *J. Steroid. Biochem.* **34**, 521–525.
- Horvath, T.L. and Wikler, K.C. (1999) Aromatase in developing sensory systems of the rat brain. *J. Neuroendocrinol.* **11**, 77–84.
- Houtsmuller, E.J. and Slob, A.K. (1990) Masculinization and defeminization of female rats by males located caudally in the uterus. *Physiol. Behav.* **48**, 555–560.
- Hoyt, K.R., Sharma, T.A. and Reynolds, I.J. (1997) Trifluoperazine and dibucaine-induced inhibition of glutamate-induced mitochondrial depolarization in rat cultured forebrain neurons. *Br. J. Pharmacol.* **122**, 803–808.
- Hu, Z.Y., Bourreau, E., Jung-Testas, I., Robel, P. and Baulieu, E.E. (1987) Neurosteroids: oligodendrocyte mitochondria convert cholesterol to pregnenolone. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8215–8219.
- Hu, Z.Y., Jung-Testas, I., Robel, P. and Baulieu, E.E. (1989) Neurosteroids: steroidogenesis in primary cultures of rat glial cells after release of aminoglutethimide blockade. *Biochem. Biophys. Res. Commun.* **161**, 917–922.
- Huang, L.S., Voyiaziakis, E., Markenson, D.F., Sokol, K.A., Hayek, T. and Breslow, J.L. (1995) Apo B gene knockout in mice results in embryonic lethality in homozygotes and neural tube defects, male infertility, and reduced HDL cholesterol ester and apo A-I transport rates in heterozygotes. *J. Clin. Invest.* **96**, 2152–2161.
- Hutchison, J.B. (1993) Aromatase: neuromodulator in the control of behavior. *J. Steroid. Biochem. Molec. Biol.* **44**, 509–520.
- Hutchison, J.B., Beyer, C., Hutchison, R.E. and Wozniak, A. (1997) Sex differences in the regulation of embryonic brain aromatase. *J. Steroid. Biochem. Molec. Biol.* **61**, 315–322.
- Hwang, P.L. (1992) Inhibitors of protein and RNA synthesis block the cytotoxic effects of oxygenated sterols. *Biochim. Biophys. Acta* **1136**, 5–11.
- Ibarrola, I., Alejandro, A., Marino, A., Sancho, M.J., Macarulla, J.M. and Trueba, M. (1992) Characterization by photoaffinity labeling of a steroid binding protein in rat liver plasma membrane. *J. Membr. Biol.* **125**, 185–191.
- Imai, A., Ohno, T. and Tamaya, T. (1992) Dehydroepiandrosterone sulfate-binding sites in plasma membrane from human uterine cervical fibroblasts. *Experientia* **48**, 999–1002.
- Inoue, K., Kubota, S. and Seyama, Y. (1999) Cholestanol induces apoptosis of cerebellar neuronal cells. *Biochem. Biophys. Res. Commun.* **256**, 198–203.



- Jaarsma, D., Postema, F. and Korf, J. (1992) Time course and distribution of neuronal degeneration in the dentate gyrus of rat after adrenalectomy: a silver impregnation study. *Hippocampus* **2**, 143–150.
- Jacobson, L. and Sapolsky, R. (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. *Endocr. Rev.* **12**, 118–134.
- Jaffuel, D., Demoly, P., Gougat, C., Mautino, G., Bousquet, J. and Mathieu, M. (1999) Rifampicin is not an activator of the glucocorticoid receptor in A549 human alveolar cells. *Mol. Pharmacol.* **55**, 841–846.
- Jamieson, P.M., Chapman, K.E., Edwards, C.R. and Seckl, J.R. (1995) 11 $\beta$ -hydroxysteroid dehydrogenase is an exclusive 11 $\beta$ -reductase in primary cultures of rat hepatocytes: effect of physico-chemical and hormonal manipulations. *Endocrinology* **136**, 4754–4761.
- Jamieson, P.M., Fuchs, E., Flugge, G. and Seckl, J.R. (1997) Attenuation of hippocampal 11 $\beta$ -hydroxy-steroid dehydrogenase type 1 by chronic psychosocial stress in the tree shrew. *Stress* **2**, 123–132.
- Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R. and Mangelsdorf, D.J. (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR $\alpha$ . *Nature* **383**, 728–731.
- Jbilo, O., Vidal, H., Paul, R., De Nys, N., Bensaid, M., Silve, S., Carayon, P., Davi, D., Galiegue, S., Bourrie, B., Guillemot, J.C., Ferrara, P., Loison, G., Maffrand, J.P., Le Fur, G. and Casellas, P. (1997) Purification and characterization of the human SR 31747A-binding protein. A nuclear membrane protein related to yeast sterol isomerase. *J. Biol. Chem.* **272**, 27107–27115.
- Jellinck, P.M., Pavlides, C., Sakai, R.R. and McEwen, B.S. (1999) 11 $\beta$ -hydroxysteroid dehydrogenase functions reversibly as an oxidoreductase in the rat hippocampus *in vivo*. *J. Steroid. Biochem. Molec. Biol.* **71**, 139–144.
- Jimbo, M., Okubo, K., Toma, Y., Shimizu, Y., Saito, H. and Yanaihara, T. (1998) Inhibitory effects of catecholamines and maternal stress on aromatase activity in the fetal rat brain. *J. Obstet. Gynaecol. Res.* **24**, 291–297.
- Jo, D.H., Abdallah, M.A., Young, J., Baulieu, E.E. and Robel, P. (1989) Pregnenolone, dehydroepiandrosterone, and their sulfate and fatty acid esters in the rat brain. *Steroids* **54**, 287–297.
- Johnson, B.H., Ayala-Torres, S., Chan, L.N., El-Naghy, M. and Thompson, E.B. (1997) Glucocorticoid/oxysterol-induced DNA lysis in human leukemic cells. *J. Steroid. Biochem. Molec. Biol.* **61**, 35–45.
- Johnson, R.L. and Scott, M.P. (1998) New players and puzzles in the Hedgehog signaling pathway. *Curr. Opin. Genet. Dev.* **8**, 450–456.
- Joliot, A., Pernelle, C., Deagostini-Bazin, H. and Prochiantz, A. (1991) Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1864–1868.
- Jones, P.B. and Hsueh, A.J. (1982) Pregnenolone biosynthesis by cultured rat granulosa cells: modulation by follicle-stimulating hormone and gonadotropin-releasing hormone. *Endocrinology* **111**, 713–721.
- Jubiz, W. and Meikle, A.W. (1979) Alterations of glucocorticoid actions by other drugs and disease states. *Drugs* **18**, 113–121.
- Jung-Testas, I., Hu, Z.Y., Baulieu, E.E. and Robel, P. (1989) Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. *Endocrinology* **125**, 2083–2091.
- Jurevics, H., Bouldin, T.W., Toews, A.D. and Morell, P. (1998) Regenerating sciatic nerve does not utilize circulating cholesterol. *Neurochem. Res.* **23**, 401–406.
- Jurevics, H. and Morell, P. (1995) Cholesterol for synthesis of myelin is made locally, not imported into brain. *J. Neurochem.* **64**, 895–901.

- Kabbadj, K., el-Etr, M., Baulieu, E.E. and Robel, P. (1993) Pregnenolone metabolism in rodent embryonic neurons and astrocytes. *Glia* **7**, 170–175.
- Kalimi, M., Shafagoj, Y., Loria, R., Padgett, D. and Regelson, W. (1994) Anti-glucocorticoid effects of dehydroepiandrosterone (DHEA). *Mol. Cell. Biochem.* **131**, 99–104.
- Kalmijn, S., Launer, L.J., Stolk, R.P., de Jong, F.H., Pols, H.A., Hofman, A., Breteler, M.M. and Lamberts, S.W. (1998) A prospective study on cortisol, dehydroepiandrosterone sulfate, and cognitive function in the elderly. *J. Clin. Endocrinol. Metab.* **83**, 3487–3492.
- Kaminska, M., Harris, J., Gijsbers, K. and Dubrovsky, B. (2000) Dehydroepiandrosterone sulfate (DHEAS) counteracts decremental effects of corticosterone on dentate gyrus LTP. Implications for depression. *Brain Res. Bull.* **52**, 229–234.
- Kang, D.E., Saitoh, T., Chen, X., Xia, Y., Masliah, E., Hansen, L.A., Thomas, R.G., Thai, L.J. and Katzman, R. (1997) Genetic association of the low-density lipoprotein receptor-related protein gene (LRP), an apolipoprotein E receptor, with late-onset Alzheimer's disease. *Neurology* **49**, 56–61.
- Kaplan, J.R., Muldoon, M.F., Manuck, S.B. and Mann, J.J. (1997) Assessing the observed relationship between low cholesterol and violence-related mortality. Implications for suicide risk. *Ann. NY Acad. Sci.* **836**, 57–80.
- Kato, J., Yamada-Mouri, N. and Hirata, S. (1997) Structure of aromatase mRNA in the rat brain. *J. Steroid. Biochem. Molec. Biol.* **61**, 381–385.
- Keddie, K.M. (1987) Severe depressive illness in the context of hypervitaminosis D. *Br. J. Psychiatry* **150**, 394–396.
- Keeney, D.S., Ikeda, Y., Waterman, M.R. and Parker, K.L. (1995) Cholesterol side-chain cleavage cytochrome P450 gene expression in the primitive gut of the mouse embryo does not require steroidogenic factor 1. *Mol. Endocrinol.* **9**, 1091–1098.
- Kim, Y.S., Zhang, H. and Kim, H.Y. (2000) Profiling neurosteroids in cerebrospinal fluids and plasma by gas chromatography/electron capture negative chemical ionization mass spectrometry. *Analyt. Biochem.* **277**, 187–195.
- Kimionides, V.G., Spillantini, M.G., Sofroniew, M.V., Fawcett, J.W. and Herbert, J. (1999) Dehydroepiandrosterone antagonizes the neurotoxic effects of corticosterone and translocation of stress-activated protein kinase 3 in hippocampal primary cultures. *Neuroscience* **89**, 429–436.
- Klette, K.L., DeCoster, M.A., Moreton, J.E. and Tortella, F.C. (1995) Role of calcium in sigma-mediated neuroprotection in rat primary cortical neurons. *Brain Res.* **704**, 31–41.
- Kobayashi, K., Ohno, S., Shinoda, M., Toyoshima, S. and Nakajin, S. (1996) Immunochemical distribution and immunohistochemical localization of 20 $\beta$ -hydroxysteroid dehydrogenase in neonatal pig tissues. *J. Steroid. Biochem. Molec. Biol.* **59**, 485–493.
- Koenig, H.L., Schumacher, M., Ferzaz, B., Thi, A.N., Ressouches, A., Guennoun, R., Jung-Testas, I., Robel, P., Akwa, Y. and Baulieu, E.E. (1995) Progesterone synthesis and myelin formation by Schwann cells. *Science* **268**, 1500–1503.
- Kohchi, C., Ukena, K. and Tsutsui, K. (1998) Age- and region-specific expressions of the messenger RNAs encoding for steroidogenic enzymes P450scc, P450c17 and 3 $\beta$ -HSD in the postnatal rat brain. *Brain Res.* **801**, 233–238.
- Kokate, T.G., Banks, M.K., Magee, T., Yamaguchi, S. and Rogawski, M.A. (1999) Finasteride, a 5 $\alpha$ -reductase inhibitor, blocks the anticonvulsant activity of progesterone in mice. *J. Pharmacol. Exp. Ther.* **288**, 679–684.
- Kolsch, H., Ludvig, M., Lutjohann, D., Prange, W. and Rao, M.L. (2000) 7 $\alpha$ -Hydroperoxycholesterol causes CNS neuronal cell death. *Neurochem. Int.* **36**, 507–512.
- Kolsch, H., Lutjohann, D., Tulke, A., Björkhem, I. and Rao, M.L. (1999) The neurotoxic effect of 24-hydroxycholesterol on SH-SY5Y human neuroblastoma cells. *Brain Res.* **818**, 171–175.

- Korneyev, A., Pan, B.S., Polo, A., Romeo, E., Guidotti, A. and Costa, E. (1993) Stimulation of brain pregnenolone synthesis by mitochondrial diazepam binding inhibitor receptor ligands *in vivo*. *J. Neurochem.* **61**, 1515–1524.
- Kotelevtsev, Y., Brown, R.W., Fleming, S., Kenyon, C., Edwards, C.R., Seckl, J.R. and Mullins, J.J. (1999) Hypertension in mice lacking 11 $\beta$ -hydroxysteroid dehydrogenase type 2. *J. Clin. Invest.* **103**, 683–689.
- Kroboth, P.D., Salek, F.S., Stone, R.A., Bertz, R.J. and Kroboth, III, F.J. (1999) Alprazolam increases dehydroepiandrosterone concentrations. *J. Clin. Psychopharmacol.* **19**, 114–124.
- Kumar, R., Thompson, E.B., Shibata, H., Spencer, T.E., Onate, S.A., Jenster, G., Tsai, S.Y., Tsai, M.J. and O'Malley, B.W. (1997) The structure of the nuclear hormone receptors Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Prog. Horm. Res.* **52**, 141–165.
- Labit-Le Bouteiller, C., Jamme, M.F., David, M., Silve, S., Lanau, C., Dhers, C., Picard, C., Rahier, A., Taton, M., Loison, G., Caput, D., Ferrara, P. and Lupker, J. (1998) Antiproliferative effects of SR31747A in animal cell lines are mediated by inhibition of cholesterol biosynthesis at the sterol isomerase step. *Eur. J. Biochem.* **256**, 342–349.
- Lackner, C., Daufeldt, S., Wildt, L. and Allera, A. (1998) Glucocorticoid-recognizing and -effector sites in rat liver plasma membrane. Kinetics of corticosterone uptake by isolated membrane vesicles. III. Specificity and stereospecificity. *J. Steroid. Biochem. Molec. Biol.* **64**, 69–82.
- Lafaye, P., Chmielewski, V., Nato, F., Mazie, J.C. and Morfin, R. (1999) The 7 $\alpha$ -hydroxysteroids produced in human tonsils enhance the immune response to tetanus toxoid and Bordetella pertussis antigens. *Biochim. Biophys. Acta* **1472**, 222–231.
- Laitinen, S., Olkkonen, V.M., Ehnholm, C. and Ikonen, E. (1999) Family of human oxysterol binding protein (OSBP) homologues. A novel member implicated in brain sterol metabolism. *J. Lipid Res.* **40**, 2204–2211.
- Lambert, J.J., Belelli, D., Hill-Venning, C., Callachan, H. and Peters, J.A. (1996) Neurosteroid modulation of native and recombinant GABAA receptors. *Cell. Mol. Neurobiol.* **16**, 155–174.
- Lambert, J.J., Belelli, D., Hill-Yenning, C. and Peters, J.A. (1995) Neurosteroids and GABA<sub>A</sub> receptor function. *Trends Pharmacol. Sci.* **16**, 295–303.
- Langan, T.J. and Volpe, J.J. (1987) Cell cycle-specific requirement for mevalonate, but not for cholesterol, for DNA synthesis in glial primary cultures. *J. Neurochem.* **49**, 513–521.
- Lange, Y. and Steck, T.L. (1998) Four cholesterol-sensing proteins. *Curr. Opin. Struct. Biol.* **8**, 435–439.
- Lanoue, L., Dehart, D.B., Hinsdale, M.E., Maeda, N., Tint, G.S. and Sulik, K.K. (1997) Limb, genital, CNS, and facial malformations result from gene/environment-induced cholesterol deficiency: further evidence for a link to sonic hedgehog. *Am. J. Med. Genet.* **73**, 24–31.
- Lansdowne, A.T. and Provost, S.C. (1998) Vitamin D<sub>3</sub> enhances mood in healthy subjects during winter. *Psychopharmacology (Berl)* **135**, 319–323.
- Lathe, R. (2001) Hormones and the hippocampus. *J. Endocrinol.* In press.
- Laubert, M.E. and Lichtensteiger, W. (1996) Ontogeny of 5 $\alpha$ -reductase (type 1) messenger ribonucleic acid expression in rat brain: early presence in germinal zones. *Endocrinology* **137**, 2718–2730.
- Laudet, V. (1997) Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J. Mol. Endocrinol.* **19**, 207–226.
- Laudet, V., Hanni, C., Coll, J., Catzeflis, F. and Stehelin, D. (1992) Evolution of the nuclear receptor gene superfamily. *EMBO J.* **11**, 1003–1013.

- Lavallee, B., Provost, P.R., Roy, R., Gauthier, M.C. and Belanger, A. (1996) Dehydroepiandrosteronefatty acid esters in human plasma: formation, transport and delivery to steroid target tissues. *J. Endocrinol.* **150**, S119–S124.
- LaVoie, H.A. and Witorsch, R.J. (1995) Investigation of intracellular signals mediating the antiapoptotic action of prolactin in Nb2 lymphoma cells. *Proc. Soc. Exp. Biol. Med.* **209**, 257–269.
- Le Goascogne, C., Robel, P., Guezou, M., Sananes, N., Baulieu, E.E. and Waterman, M. (1987) Neurosteroids: cytochrome P-450scc in rat brain. *Science* **237**, 1212–1215.
- Le Goascogne, C., Sananes, N., Guezou, M., Takemori, S., Kominami, S., Baulieu, E.E. and Robel, P. (1991) Immunoreactive cytochrome P-450 (17 $\alpha$ ) in rat and guinea-pig gonads, adrenal glands and brain. *J. Reprod. Fertil.* **93**, 609–622.
- Lehmann, J.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A. and Willson, T.M. (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J. Biol. Chem.* **272**, 3137–3140.
- Leighton, J.K., Dueland, S., Straka, M.S., Trawick, J. and Davis, R.A. (1991) Activation of the silent endogenous cholesterol-7- $\alpha$ -hydroxylase gene in rat hepatoma cells: a new complementation group having resistance to 25-hydroxycholesterol. *Mol. Cell. Biol.* **11**, 2049–2056.
- Leitersdorf, E., Reshef, A., Meiner, V., Levitzki, R., Schwartz, S.P., Dann, E.J., Berkman, N., Cali, J.J., Klapholz, L. and Berginer, V.M. (1993) Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in Jews or Moroccan origin. *J. Clin. Invest.* **91**, 2488–2496.
- Lemaire, S., Lizard, G., Monier, S., Miguet, C., Gueldry, S., Volot, F., Gambert, P. and Neel, D. (1998) Different patterns of IL-1 $\beta$  secretion, adhesion molecule expression and apoptosis induction in human endothelial cells treated with 7 $\alpha$ -, 7 $\beta$ -hydroxycholesterol, or 7-ketocholesterol. *FEBS Lett.* **440**, 434–439.
- Lephart, E.D. (1997) Molecular aspects of brain aromatase cytochrome P450. *J. Steroid. Biochem. Molec. Biol.* **61**, 375–380.
- Li, K., Foo, T. and Adams, J.B. (1978) Products of dehydroepiandrosterone metabolism by human mammary tumors and their influence on estradiol receptor binding. *Steroids* **31**, 113–127.
- Li, X., Bertics, P.J. and Karavolas, H.J. (1997) Regional distribution of cytosolic and particulate 5 $\alpha$ -dihydroprogesterone 3 $\alpha$ -hydroxysteroid oxidoreductases in female rat brain. *J. Steroid. Biochem. Molec. Biol.* **60**, 311–318.
- Li-Hawkins, J., Lund, E.G., Bronson, A.D. and Russell, D.W. (2000) Expression cloning of an oxysterol 7 $\alpha$ -hydroxylase selective for 24-hydroxycholesterol. *J. Biol. Chem.* **275**, 16543–16549.
- Lin, H.K., Jez, J.M., Schlegel, B.P., Peehl, D.M., Pachter, J.A. and Penning, T.M. (1997) Expression and characterization of recombinant type 2 3 $\alpha$ -hydroxysteroid dehydrogenase (HSD) from human prostate: demonstration of bifunctional 3 $\alpha$ /17 $\beta$ -HSD activity and cellular distribution. *Mol. Endocrinol.* **11**, 1971–1984.
- Liscum, L. and Munn, N.J. (1999) Intracellular cholesterol transport. *Biochim. Biophys. Acta* **1438**, 19–37.
- Lizard, G., Deckert, V., Dubrez, L., Moisan, M., Gambert, P. and Lagrost, L. (1996) Induction of apoptosis in endothelial cells treated with cholesterol oxides. *Am. J. Pathol.* **148**, 1625–1638.
- Lizard, G., Lemaire, S., Monier, S., Gueldry, S., Neel, D. and Gambert, P. (1997) Induction of apoptosis and of interleukin-1 $\beta$  secretion by 7 $\beta$ -hydroxycholesterol and 7-ketocholesterol: partial inhibition by Bcl-2 overexpression. *FEBS Lett.* **419**, 276–280.
- Loria, R.M. (1997) Antigluco-corticoid function of androstenetriol. *Psychoneuroendocrinology* **22**, S103–S108.

- Loria, R.M., Padgett, D.A. and Huynh, P.N. (1996) Regulation of the immune response by dehydroepiandrosterone and its metabolites. Regulation of the immune response by dehydroepiandrosterone and its metabolites. *J. Endocrinol.* **150**, S209–S220.
- Lund, E.G., Guileyardo, J.M. and Russell, D.W. (1999) cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7238–7243.
- Lund, E.G., Kerr, T.A., Sakai, J., Li, W.P. and Russell, D.W. (1998) cDNA cloning of mouse and human cholesterol 25-hydroxylases, polytopic membrane proteins that synthesize a potent oxysterol regulator of lipid metabolism. *J. Biol. Chem.* **273**, 34316–34327.
- Lutjohann, D., Breuer, O., Ahlborg, G., Nennesmo, I., Siden, A., Diczfalussy, U. and Björkhem, I. (1996) Cholesterol homeostasis in human brain: evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 9799–9804.
- Lutjohann, D., Papassotiropoulos, A., Björkhem, I., Locatelli, S., Bagli, M., Oehring, R.D., Schlegel, U., Jessen, F., Rao, M.L., von Bergmann, K. and Heun, R. (2000) Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients. *J. Lipid Res.* **41**, 195–198.
- MacKenzie, S.M., Clark, C.J., Eraser, R., Gornes-Sanchez, C.E., Connell, J.M. and Davies, E. (2000) Expression of 11 $\beta$ -hydroxylase and aldosterone synthase genes in the rat brain. *J. Mol. Endocrinol.* **24**, 321–328.
- MacLaughlin, J. and Holick, M.F. (1985) Aging decreases the capacity of human skin to produce vitamin D<sub>3</sub>. *J. Clin. Invest.* **76**, 1536–1538.
- MacLusky, N.J. and Naftolin, F. (1981) Sexual differentiation of the central nervous system. *Science* **211**, 1294–1302.
- Maitra, R. and Reynolds, J.N. (1998) Modulation of GABA<sub>A</sub> receptor function by neuroactive steroids: evidence for heterogeneity of steroid sensitivity of recombinant GABA<sub>A</sub> receptor isoforms. *Can. J. Physiol. Pharmacol.* **76**, 909–920.
- Majewska, M.D. (1992) Neurosteroids: endogenous bimodal modulators of the GABA<sub>A</sub> receptor. Mechanism of action and physiological significance. *Prog. Neurobiol.* **38**, 379–395.
- Majewska, M.D., Demigoren, S., Spivak, C.E. and London, E.D. (1990) The neurosteroid dehydroepiandrosterone sulfate is an allosteric antagonist of the GABA<sub>A</sub> receptor. *Brain Res.* **526**, 143–146.
- Majewska, M.D., Harrison, N.L., Schwartz, R.D., Barker, J.L. and Paul, S.M. (1986) Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* **232**, 1004–1007.
- Makishima, M., Okamoto, A.Y., Repa, J.J., Tu, H., Learned, R.M., Luk, A., Hull, M.V., Lustig, K.D., Mangelsdorf, D.J. and Shan, B. (1999) Identification of a nuclear receptor for bile acids. *Science* **284**, 1362–1365.
- Marrapodi, M. and Chiang, J.Y. (2000) Peroxisome proliferator-activated receptor alpha (PPARalpha) and agonist inhibit cholesterol 7 $\alpha$ -hydroxylase gene (CYP7A1) transcription. *J. Lipid Res.* **41**, 514–520.
- Martin, K.O., Reiss, A.B., Lathe, R. and Javitt, N.B. (1997) 7 $\alpha$ -hydroxylation of 27-hydroxycholesterol: biologic role in the regulation of cholesterol synthesis. *J. Lipid Res.* **38**, 1053–1058.
- Maschler, L., Salzberger, M. and Finkelstein, M. (1975) 11 $\beta$ -Hydroxylase with affinity to C-21Deoxysteroids from ovaries of patients with polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* **41**, 999–1002.

- Mason, J.I., Keeney, D.S., Bird, I.M., Rainey, W.E., Morohashi, K., Leers-Sucheta, S. and Melner, M.H. (1997) The regulation of  $3\beta$ -hydroxysteroid dehydrogenase expression. *Steroids* **62**, 164–168.
- Masters, J.N., Finch, C.E. and Sapolsky, R.M. (1989) Glucocorticoid endangerment of hippocampal neurons does not involve deoxyribonucleic acid cleavage. *Endocrinology* **124**, 3083–3088.
- Matheson, G.K., Guthrie, D., Bauer, C., Knowles, A., White, G. and Ruston, C. (1991) Sigma receptor ligands alter concentrations of corticosterone in plasma in the rat. *Neuropharmacology* **30**, 79–87.
- Matsuura, K., Shiraishi, H., Hara, A., Sato, K., Deyashiki, Y., Ninomiya, M. and Sakai, S. (1998) Identification of a principal mRNA species for human  $3\alpha$ -hydroxysteroid dehydrogenase isoform (AKR1C3) that exhibits high prostaglandin D<sub>2</sub> 11-ketoreductase activity. *J. Biochem. (Tokyo)* **124**, 940–946.
- Maurice, T., Phan, V.L., Urani, A., Kamei, H., Noda, Y. and Nabeshima, T. (1999) Neuroactive neurosteroids as endogenous effectors for the sigma1 (sigma1) receptor: pharmacological evidence and therapeutic opportunities. *Jpn. J. Pharmacol.* **81**, 125–155.
- Maurice, T., Su, T.P. and Privat, A. (1998) Sigma1 (sigma 1) receptor agonists and neurosteroids attenuate B25–35-amyloid peptide-induced amnesia in mice through a common mechanism. *Neuroscience* **83**, 413–428.
- May, M., Holmes, E., Rogers, W. and Poth, M. (1990) Protection from glucocorticoid induced thymic involution by dehydroepiandrosterone. *Life Sci.* **46**, 1627–1631.
- McCauley, L.D., Park, C.H., Lan, N.C., Tomich, J.M., Shively, J.E. and Gee, K.W. (1995) Benzodiazepines and peptides stimulate pregnenolone synthesis in brain mitochondria. *Eur. J. Pharmacol.* **276**, 145–153.
- McDonald, J.W., Goldberg, M.P., Gwag, B.J., Chi, S.I. and Choi, D.W. (1996) Cyclosporine induces neuronal apoptosis and selective oligodendrocyte death in cortical cultures. *Ann. Neurol.* **40**, 750–758.
- McEnery, M.W., Dawson, T.M., Verma, A., Gurley, D., Colombini, M. and Snyder, S.H. (1993) Mitochondrial voltage-dependent anion channel. Immunochemical and immunohistochemical characterization in rat brain. *J. Biol. Chem.* **268**, 23289–23296.
- McEnery, M.W., Snowman, A.M., Trifiletti, R.R. and Snyder, S.H. (1992) Isolation of the mitochondrial benzodiazepine receptor: association with the voltage-dependent anion channel and the adenine nucleotide carrier. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3170–3174.
- McEwen, B.S. (1999) Stress and the aging hippocampus. *Front. Neuroendocrinol.* **20**, 49–70.
- McEwen, B.S. and Alves, S.E. (1999) Estrogen actions in the central nervous system. *Endocr. Rev.* **20**, 279–307.
- McEwen, B.S., Lambdin, L.T., Rainbow, T.C. and De Nicola, A.F. (1986) Aldosterone effects on salt appetite in adrenalectomized rats. *Neuroendocrinology* **43**, 38–43.
- McIntosh, M., Bao, H. and Lee, C. (1999) Opposing actions of dehydroepiandrosterone and corticosterone in rats. *Proc. Soc. Exp. Biol. Med.* **221**, 198–206.
- McNamara, R.K. and Skelton, R.W. (1991) Diazepam impairs acquisition but not performance in the Morris water maze. *Pharmacol. Biochem. Behav.* **38**, 651–658.
- McNamara, R.K. and Skelton, R.W. (1992) Like diazepam, CL 218, 872, a selective ligand for the benzodiazepine omega 1 receptor subtype, impairs place learning in the Morris water maze. *Psychopharmacology* **107**, 347–351.
- Meaney, S., Lutjohann, D., Diczfalussy, U. and Björkhem, I. (2000) Formation of oxysterols from different pools of cholesterol as studied by stable isotope technique: cerebral origin of most

- circulating 24S-hydroxycholesterol in rats, but not in mice. *Biochim. Biophys. Acta* **1486**, 293–298.
- Mehtali, M. (1988) *PhD Thesis*, University of Strasbourg, France.
- Mehtali, M., LeMeur, M. and Lathe, R. (1990) The methylation-free status of a housekeeping transgene is lost at high copy number. *Gene* **91**, 179–184.
- Meikle, A.W., Dorchuck, R.W., Araneo, B.A., Stringham, J.D., Evans, T.G., Spruance, S.L. and Daynes, R.A. (1992) The presence of a dehydroepiandrosterone-specific receptor binding complex in murine T cells. *J. Steroid. Biochem. Molec. Biol.* **42**, 293–304.
- Melcangi, R.C., Celotti, F., Castano, P. and Martini, L. (1993) Differential localization of the 5 $\alpha$ -reductase and the 3 $\alpha$ -hydroxysteroid dehydrogenase in neuronal and glial cultures. *Endocrinology* **132**, 1252–1259.
- Melcangi, R.C., Poletti, A., Cavarretta, L., Celotti, F., Colciago, A., Magnaghi, V., Motta, M., NegriCesi, P. and Martini, L. (1998) The 5 $\alpha$ -reductase in the central nervous system: expression and modes of control. *J. Steroid. Biochem. Molec. Biol.* **65**, 295–299.
- Mellon, W.S. (1984) Inhibitory action of aurointricarboxylic acid and rifamycin AF/013 at the polynucleotide domain of 1,25-dihydroxyvitamin D<sub>3</sub>-receptor complexes. *Biochem. Pharmacol.* **33**, 1047–1057.
- Mensah-Nyagan, A.G., Do-Rego, J.L., Beaujean, D., Luu-The, V., Pelletier, G. and Vaudry, H. (1999) Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacol. Rev.* **51**, 63–81.
- Meresse, S., Delbart, C., Fruchart, J.C. and Cecchelli, R. (1989) Low-density lipoprotein receptor on endothelium of brain capillaries. *J. Neurochem.* **53**, 340–345.
- Michikawa, M. and Yanagisawa, K. (1999) Inhibition of cholesterol production but not of nonsterol isoprenoid products induces neuronal cell death. *J. Neurochem.* **72**, 2278–2285.
- Miyake, J.H., Wang, S.L. and Davis, R.A. (2000) Bile acid induction of cytokine expression by macrophages correlates with repression of hepatic cholesterol 7 $\alpha$ -hydroxylase. *J. Biol. Chem.* **275**, 21805–21808.
- Miyamoto, H., Yeh, S., Lardy, H., Messing, E. and Chang, C. (1998)  $\Delta^5$ -androstenediol is a natural hormone with androgenic activity in human prostate cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 11083–11088.
- Moebius, F.F., Bermoser, K., Reiter, R.J., Manner, M. and Glossmann, H. (1996) Yeast sterol C8-C7 isomerase: identification and characterization of a high-affinity binding site for enzyme inhibitors. *Biochemistry* **35**, 16871–16878.
- Moebius, F.F., Fitzky, B.U., Lee, J.N., Paik, Y.K. and Glossmann, H. (1998) Molecular cloning and expression of the human  $\Delta^7$ -sterol reductase. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1899–1902.
- Moebius, F.F., Reiter, R.J., Manner, M. and Glossmann, H. (1997a) High affinity of sigma 1-binding sites for sterol isomerization inhibitors: evidence for a pharmacological relationship with the yeast sterol C8-C7 isomerase. *Br. J. Pharmacol.* **121**, 1–6.
- Moebius, F.F., Striessnig, J. and Glossmann, H. (1997b) The mysteries of sigma receptors: new family members reveal a role in cholesterol synthesis. *Trends Pharmacol. Sci.* **18**, 67–70.
- Moffat, S.D., Zonderman, A.B., Harman, S.M., Blackman, M.R., Kawas, C. and Resnick, S.M. (2000) The relationship between longitudinal declines in dehydroepiandrosterone sulfate concentrations and cognitive performance in older men. *Arch. Intern. Med.* **160**, 2193–2198.
- Moisan, M.P., Edwards, C.R. and Seckl, J.R. (1992) Ontogeny of 11 $\beta$ -hydroxysteroid dehydrogenase in rat brain and kidney. *Endocrinology* **130**, 400–404.
- Moisan, M.P., Seckl, J.R. and Edwards, C.R. (1990) 11 $\beta$ -hydroxysteroid dehydrogenase bioactivity and messenger RNA expression in rat forebrain: localization in hypothalamus, hippocampus, and cortex. *Endocrinology* **127**, 1450–1455.

- Monje, P. and Boland, R. (1999) Characterization of membrane estrogen binding proteins from rabbit uterus. *Mol. Cell. Endocrinol.* **147**, 75–84.
- Monnet, F.P., Mahe, V., Robel, P. and Baulieu, E.E. (1995) Neurosteroids, via sigma receptors, modulate the [<sup>3</sup>H]norepinephrine release evoked by N-methyl-D-aspartate in the rat hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3774–3778.
- Moog, C., Aubertin, A.M., Kirn, A. and Luu, B. (1998) Oxysterols, but not cholesterol, inhibit human immunodeficiency virus replication *in vitro*. *Antivir. Chem. Chemother.* **9**, 491–496.
- Moog, C., Deloulme, J.C., Baudier, J., Revel, M.O., Bischoff, P., Hietter, H. and Luu, B. (1991) Membrane-related oxysterol function: preliminary results on the modification of protein kinase C activity and substrate phosphorylation by 7-beta, 25-dihydroxycholesterol. *Biochimie* **73**, 1321–1326.
- Moore, F.L. and Orchinik, M. (1994) Membrane receptors for corticosterone: a mechanism for rapid behavioral responses in an amphibian. *Horm. Behav.* **28**, 512–519.
- Morales, A.J., Nolan, J.J., Nelson, J.C. and Yen, S.S. (1994) Effects of replacement dose of dehydroepiandrosterone in men and women of advancing age. *J. Clin. Endocrinol. Metab.* **78**, 1360–1367.
- Morell, P. and Jurevics, H. (1996) Origin of cholesterol in myelin. *Neurochem. Res.* **21**, 463–470.
- Morfin, R. and Courchay, G. (1994) Pregnenolone and dehydroepiandrosterone as precursors of native 7-hydroxylated metabolites which increase the immune response in mice. *J. Steroid. Biochem. Molec. Biol.* **50**, 91–100.
- Morfin, R.F., Robel, P. and Baulieu, E.E. (1992a) Neurosteroid metabolism: 7 $\alpha$ -hydroxylation of dehydroepiandrosterone and pregnenolone by rat brain microsomes. *Biochem. J.* **288**, 959–964.
- Morfin, R., Young, J., Corpechot, C., Egestad, B., Sjoval, J. and Baulieu, E.E. (1992b) Neurosteroids: pregnenolone in human sciatic nerves. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6790–6793.
- Mune, T., Rogerson, F.M., Nikkila, H., Agarwal, A.K. and White, P.C. (1995) Human hypertension caused by mutations in the kidney isozyme of 11 $\beta$ -hydroxysteroid dehydrogenase. *Nat. Genet.* **10**, 394–399.
- Murphy, B.E. (1981) Demonstration of novel compounds in human fetal tissues and a consideration of their possible role in parturition. *Am. J. Obstet. Gynecol.* **139**, 353–358.
- Murphy, B.E. (1991) Steroids and depression. *J. Steroid. Biochem. Molec. Biol.* **38**, 537–559.
- Murphy, B.E. (1997) Antigluco-corticoid therapies in major depression: a review. *Psychoneuroendocrinology* **22S1**, 125–132.
- Myers, A.M., Charifson, P.S., Owens, C.E., Kula, N.S., McPhail, A.T., Baldessarini, R.J., Booth, R.G. and Wyrick, S.D. (1994) Conformational analysis, pharmacophore identification, and comparative molecular field analysis of ligands for the neuromodulatory sigma 3 receptor. *J. Med. Chem.* **37**, 4109–4117.
- Myers-Payne, S.C., Fontaine, R.N., Loeffler, A., Pu, L., Rao, A.M., Kier, A.B., Wood, W.G. and Schroeder, F. (1996) Effects of chronic ethanol consumption on sterol transfer proteins in mouse brain. *J. Neurochem.* **66**, 313–320.
- Navarro-Ruiz, A., Garcia-Estrada, J., Almodovar-Cuevas, C., Bastidas-Ramirez, B.E., RomanMaldonado, S. and Garzon, P. (1987) Progesterone fate in rabbit cornea. *Comp. Biochem. Physiol. [B]* **86**, 607–611.
- Ness, G.C. (1994) Developmental regulation of the expression of genes encoding proteins involved in cholesterol homeostasis. *Am. J. Med. Genet.* **50**, 355–357.
- Ness, G.C., Miller, J.P., Moffler, M.H., Woods, L.S. and Harris, H.B. (1979) Perinatal development of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in rat lung, liver and brain. *Lipids* **14**, 447–450.



- Neve, E.P. and Ingelman-Sundberg, M. (1999) A soluble NH(2)-terminally truncated catalytically active form of rat cytochrome P450 2E1 targeted to liver mitochondria. *FEBS Lett.* **460**, 309–314.
- Nishio, E., Arimura, S. and Watanabe, Y. (1996) Oxidized LDL induces apoptosis in cultured smooth muscle cells: a possible role for 7-ketocholesterol. *Biochem. Biophys. Res. Commun.* **223**, 413–418.
- Nitta, M., Ku, S., Brown, C., Okamoto, A.Y. and Shan, B. (1999) CPF: an orphan nuclear receptor that regulates liver-specific expression of the human cholesterol 7 $\alpha$ -hydroxylase gene. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6660–6665.
- Norlin, M. and Wikvall, K. (1998) Biochemical characterization of the 7 $\alpha$ -hydroxylase activities towards 27-hydroxycholesterol and dehydroepiandrosterone in pig liver microsomes. *Biochim. Biophys. Acta* **1390**, 269–281.
- Nowaczyk, M.J., Whelan, D.T., Heshka, T.W. and Hill, R.E. (1999) Smith-Lemli-Opitz syndrome: a treatable inherited error of metabolism causing mental retardation. *CMAJ* **161**, 165–170.
- Nunez, S.B., Medin, J.A., Braissant, O., Kemp, L., Wahli, W., Ozato, K. and Segars, J.H. (1997) Retinoid X receptor and peroxisome proliferator-activated receptor activate an estrogen responsive gene independent of the estrogen receptor. *Molec. Cell. Endocrinol.* **127**, 27–40.
- Ogawa, S., Washburn, T.F., Taylor, J., Lubahn, D.B., Korach, K.S. and Pfaff, D.W. (1998) Modifications of testosterone-dependent behaviors by estrogen receptor- $\alpha$  gene disruption in male mice. *Endocrinology* **139**, 5058–5069.
- Ohno, S., Nakajin, S. and Shinoda, M. (1991) 20 $\beta$ -hydroxysteroid dehydrogenase of neonatal pig testis: 3  $\alpha$ /beta-hydroxysteroid dehydrogenase activities catalyzed by highly purified enzyme. *J. Steroid. Biochem. Molec. Biol.* **38**, 787–794.
- Okabe, T., Haji, M., Takayanagi, R., Adachi, M., Imasaki, K., Kurimoto, F., Watanabe, T. and Nawata, H. (1995) Up-regulation of high-affinity dehydroepiandrosterone binding activity by dehydroepiandrosterone in activated human T lymphocytes. *J. Clin. Endocrinol. Metab.* **80**, 2993–2996.
- Okuda, K.I. (1994) Liver mitochondrial P450 involved in cholesterol catabolism and vitamin D activation. *J. Lipid Res.* **35**, 361–372.
- Okuizumi, K., Onodera, O., Namba, Y., Ikeda, K., Yamamoto, T., Seki, K., Ueki, A., Nanko, S., Tanaka, H. and Takahashi, H. (1995) Genetic association of the very low density lipoprotein (VLDL) receptor gene with sporadic Alzheimer's disease. *Nat. Genet.* **11**, 207–209.
- Okuizumi, K., Onodera, O., Seki, K., Tanaka, H., Namba, Y., Ikeda, K., Saunders, A.M., Pericak-Vance, M.A., Roses, A.D. and Tsuji, S. (1996) Lack of association of very low density lipoprotein receptor gene polymorphism with Caucasian Alzheimer's disease. *Ann. Neurol.* **40**, 251–254.
- Okuyama, S., Chaki, S., Yoshikawa, R., Ogawa, S., Suzuki, Y., Okubo, T., Nakazato, A., Nagamine, M. and Tomisawa, K. (1999) Neuropharmacological profile of peripheral benzodiazepine receptor agonists, DAA1097 and DAA1106. *Life Sci.* **64**, 1455–1464.
- Olsen, K.L. (1979) Androgen-insensitive rats are defeminised by their testes. *Nature* **279**, 238–239.
- Opitz, J.M. and de la Cruz, F. (1994) Cholesterol metabolism in the RSH/Smith-Lemli-Opitz syndrome: summary of an NICHD conference. *Am. J. Med. Genet.* **50**, 326–338.
- Orentreich, N., Brind, J.L., Rizer, R.L. and Vogelman, J.H. (1984) Age changes and sex differences in serum dehydroepiandrosterone sulfate concentrations throughout adulthood. *J. Clin. Endocrinol. Metab.* **59**, 551–555.

- Orentreich, N., Brind, J.L., Vogelman, J.H., Andres, R. and Baldwin, H. (1992) Long-term longitudinal measurements of plasma dehydroepiandrosterone sulfate in normal men. *J. Clin. Endocrinol. Metab.* **75**, 1002–1004.
- Osawa, Y., Higashiyama, T., Shimizu, Y. and Yarborough, C. (1993) Multiple functions of aromatase and the active site structure; aromatase is the placental estrogen 2-hydroxylase. *J. Steroid. Biochem. Molec. Biol.* **44**, 469–480.
- Ozaki, H.S., Iwahashi, K., Tsubaki, M., Fukui, Y., Ichikawa, Y. and Takeuchi, Y. (1991) Cytochrome P-45011 beta in rat brain. *J. Neurosci. Res.* **28**, 518–524.
- Ozawa, N., Yamazaki, S., Chiba, K., Aoyama, H., Tomisawa, H., Tateishi, M. and Watabe, T. (1991) Occurrence of cholesterol 7 $\alpha$ - and 7 $\beta$ -hydroperoxides in rat skin as aging markers. *Biochem. Biophys. Res. Commun.* **178**, 242–247.
- Padgett, D.A. and Loria, R.M. (1994) *In vitro* potentiation of lymphocyte activation by dehydroepiandrosterone, androstenediol, and androstenetriol. *J. Immunol.* **153**, 1544–1552.
- Papadopoulos, V., Amri, H., Boujrad, N., Cascio, C., Culty, M., Ganier, M., Hardwick, M., Li, H., Vidic, B., Brown, A.S., Reversa, J.L., Bernassau, J.M. and Drieu, K. (1997) Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids* **62**, 21–28.
- Papadopoulos, V., Berkovich, A., Krueger, K.E., Costa, E. and Guidotti, A. (1991) Diazepam binding inhibitor and its processing products stimulate mitochondrial steroid biosynthesis via an interaction with mitochondrial benzodiazepine receptors. *Endocrinology* **129**, 1481–1488.
- Park-Chung, M., Wu, F.S., Purdy, R.H., Malayev, A.A., Gibbs, T.T. and Farb, D.H. (1997) Distinct sites for inverse modulation of N-methyl-D-aspartate receptors by sulfated steroids. *Mol. Pharmacol.* **52**, 1113–1123.
- Parks, D.J., Blanchard, S.G., Bledsoe, R.K., Chandra, G., Consler, T.G., Kliewer, S.A., Stimmel, J.B., Willson, T.M., Zavacki, A.M., Moore, D.D. and Lehmann, J.M. (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science* **284**, 1365–1368.
- Pastorino, J.G., Simbula, G., Gilfor, E., Hoek, J.B. and Farber, J.L. (1994) Protoporphyrin IX, an endogenous ligand of the peripheral benzodiazepine receptor, potentiates induction of the mitochondrial permeability transition and the killing of cultured hepatocytes by rotenone. *J. Biol. Chem.* **269**, 31041–31046.
- Payne, A.H., Abbaszade, I.G., Clarke, T.R., Bain, P.A. and Park, C.H. (1997) The multiple murine 3 beta-hydroxysteroid dehydrogenase isoforms: structure, function, and tissue- and developmentally specific expression. *Steroids* **62**, 169–175.
- Payne, D.W., Shackleton, C., Toms, H., Ben-Shlomo, I., Kol, S., deMoura, M., Strauss, J.F. and Adashi, E.Y. (1995) A novel nonhepatic hydroxycholesterol 7 $\alpha$ -hydroxylase that is markedly stimulated by interleukin-1 beta. Characterization in the immature rat ovary. *J. Biol. Chem.* **270**, 18888–18896.
- Pechnick, R.N. and Poland, R.E. (1994) Neuroendocrine responses produced by enantiomeric pairs of drugs that interact with phencyclidine and sigma receptors. *Eur. J. Pharmacol.* **263**, 115–120.
- Pedersen, J.I., Oftebro, H. and Björkhem, I. (1989) Reconstitution of C27-steroid 26-hydroxylase activity from bovine brain mitochondria. *Biochem. Int.* **18**, 615–622.
- Penning, T.M. (1997) Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr. Rev.* **18**, 281–305.
- Phan, V.L., Su, T.P., Privat, A. and Maurice, T. (1999) Modulation of steroidal levels by adrenalectomy/castration and inhibition of neurosteroid synthesis enzymes affect signal receptor-mediated behaviour in mice. *Eur. J. Neurosci.* **11**, 2385–2396.

- Pikuleva, L.A., Babiker, A., Waterman, M.R. and Björkhem, I. (1998) Activities of recombinant human cytochrome P450c27 (CYP27) which produce intermediates of alternative bile acid biosynthetic pathways. *J. Biol. Chem.* **273**, 18153–18160.
- Pocklington, T. and Jeffery, J. (1968) 3 $\alpha$ -hydroxysteroid: NAD oxidoreductase activity in crystalline preparations of 20 beta-hydroxysteroid: NAD oxidoreductase. *Eur. J. Biochem.* **7**, 63–67.
- Poirier, J., Baccichet, A., Dea, D. and Gauthier, S. (1993) Cholesterol synthesis and lipoprotein reuptake during synaptic remodelling in hippocampus in adult rats. *Neuroscience* **55**, 81–90.
- Poirier, J. and Seigny, P. (1998) Apolipoprotein E4, cholinergic integrity and the pharmacogenetics of Alzheimer's disease. *J. Neural. Transm.* **53**, (Suppl.) 199–207.
- Poletti, A. and Martini, L. (1999) Androgen-activating enzymes in the central nervous system. *J. Steroid. Biochem. Molec. Biol.* **69**, 117–122.
- Ponthier, J.L., Shackleton, C.H. and Trant, J.M. (1998) Seasonal changes in the production of two novel and abundant ovarian steroids in the channel catfish (*Ictalurus punctatus*). *Gen. Comp. Endocrinol.* **111**, 141–155.
- Porter, J.A., Young, K.E. and Beachy, P.A. (1996) Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274**, 255–259.
- Prasad, V.V., Vegesna, S.R., Welch, M. and Lieberman, S. (1994) Precursors of the neurosteroids. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3220–3223.
- Prochiantz, A. and Theodore, L. (1995) Nuclear/growth factors. *Bioessays* **17**, 39–44.
- Raber, J., Wong, D., Buttini, M., Orth, M., Bellosta, S., Pitas, R.E., Mahley, R.W. and Mucke, L. (1998) Isoform-specific effects of human apolipoprotein E on brain function revealed in Apo E knockout mice: increased susceptibility of females. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10914–10919.
- Rajan, V., Edwards, C.R. and Seckl, J.R. (1996) 11 $\beta$ -Hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. *J. Neurosci.* **16**, 65–70.
- Ramayya, M.S., Zhou, J., Kino, T., Segars, J.H., Bondy, C.A. and Chrousos, G.P. (1997) Steroidogenic factor 1 messenger ribonucleic acid expression in steroidogenic and nonsteroidogenic human tissues: northern blot and *in situ* hybridization studies. *J. Clin. Endocrinol. Metab.* **82**, 1799–1806.
- Rampe, D. and Triggle, D.J. (1987) Benzodiazepine interactions at neuronal and smooth muscle Ca<sup>2+</sup> channels. *Eur. J. Pharmacol.* **134**, 189–197.
- Rao, V.L. and Butterworth, R.F. (1997) Characterization of binding sites for the omegaS receptor ligands [3H]PK11195 and [3H]RO5-4864 in human brain. *Eur. J. Pharmacol.* **340**, 89–99.
- Richert, L., Beck, J.P. and Shinitzky, M. (1986) Inhibition of IL-2 secretion and IL-2 receptor appearance of activated lymphocytes pretreated with hydroxylated sterols. *Immunol. Lett.* **13**, 329–334.
- Rietveld, A., Neutz, S., Simons, K. and Eaton, S. (1999) Association of sterol- and glycosylphosphatidylinositol-linked proteins with Drosophila raft lipid microdomains. *J. Biol. Chem.* **274**, 12049–12054.
- Robel, P., Bourreau, E., Corpechot, C., Dang, D.C., Halberg, F., Clarke, C., Haug, M., Schlegel, M.L., Synguelakis, M. and Vourch, C. (1987) Neuro-steroids: 3 $\beta$ -hydroxy- $\Delta^5$ -derivatives in rat and monkey brain. *J. Steroid. Biochem.* **27**, 649–655.
- Robel, P., Corpechot, C., Clarke, C., Groyer, A., Synguelakis, M., Vourc'h, C. and Baulieu, E.E. (1986) Neurosteroids: 3beta-hydroxy-delta5-derivatives in the rat brain. In: G.Fink, A.J.Harmar, and K.W.McKerns (eds), *Neuroendocrine Molecular Biology*. Plenum Press, pp. 367–377

- Robertson, K.M., O'Donnell, L., Jones, M.E., Meachem, S.J., Boon, W.C., Fisher, C.R., Graves, K.H., McLachlan, R.I. and Sirmpson, E.R. (1999) Impairment of spermatogenesis in mice lacking a functional aromatase (CYP19) gene. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7986–7991.
- Robson, A.C., Leckie, C.M., Seckl, J.R. and Holmes, M.C. (1998) 11 $\beta$ -hydroxysteroid dehydrogenase type 2 in the postnatal and adult rat brain. *Brain Res. Molec. Brain Res.* **61**, 1–10.
- Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S.W., Tsui, L.C. and Muenke, M. (1996) Mutations in the human sonic hedgehog gene cause holoprosencephaly. *Nat. Genet.* **14**, 357–360.
- Romeo, E., Cavallaro, S., Korneyev, A., Kozikowski, A.P., Ma, D., Polo, A., Costa, E., Guidotti, A., McCauley, L.D., Park, C.H., Lan, N.C., Tomich, J.M., Shively, J.E. and Gee, K.W. (1995) Stimulation of brain steroidogenesis by 2-aryl-indole-3-acetamide derivatives acting at the mitochondrial diazepam-binding inhibitor receptor complex Benzodiazepines and peptides stimulate pregnenolone synthesis in brain mitochondria. *Eur. J. Pharmacol.* **276**, 145–153.
- Romeo, E., Cheney, D.L., Zivkovic, I., Costa, E. and Guidotti, A. (1994) Mitochondrial diazepam-binding inhibitor receptor complex agonists antagonize dizocilpine amnesia: putative role for allopregnanolone. *J. Pharmacol. Exp. Ther.* **270**, 89–96.
- Roscetti, G., Del Carmine, R., Trabucchi, M., Massotti, M., Purdy, R.H. and Barbaccia, M.L. (1998) Modulation of neurosteroid synthesis/accumulation by L-ascorbic acid in rat brain tissue: inhibition by selected serotonin antagonists. *J. Neurochem.* **71**, 1108–1117.
- Rose, K.A., Stapleton, G., Dott, K., Kieny, M.P., Best, R., Schwarz, M., Russell, D.W., Björkhem, I., Seckl, J. and Lathe, R. (1997) CYP7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7 $\alpha$ -hydroxydehydroepiandrosterone and 7 $\alpha$ -hydroxypregnenolone. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4925–4930.
- Roselli, C.E. and Snipes, C.A. (1984) Progesterone 5 $\alpha$ -reductase in mouse brain. *Brain Res.* **305**, 197–202.
- Rosen, H., Reshef, A., Maeda, N., Lippoldt, A., Shpizen, S., Triger, L., Eggertsen, G., Björkhem, I. and Leitersdorf, E. (1998) Markedly reduced bile acid synthesis but maintained levels of cholesterol and vitamin D metabolites in mice with disrupted sterol 27-hydroxylase gene. *J. Biol. Chem.* **273**, 14805–14812.
- Rupprecht, R., Berning, B., Hauser, C.A., Holsboer, F. and Reul, J.M. (1996) Steroid receptor-mediated effects of neuroactive steroids: characterization of structure-activity relationship. *Eur. J. Pharmacol.* **303**, 227–234.
- Rupprecht, R. and Holsboer, F. (1999) Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci.* **22**, 410–416.
- Sanger, D.J., Benavides, J., Perrault, G., Morel, E., Cohen, C., Joly, D. and Zivkovic, B. (1994) Recent developments in the behavioral pharmacology of benzodiazepine (omega) receptors: evidence for the functional significance of receptor subtypes. *Neurosci. Biobehav. Rev.* **18**, 355–372.
- Sanne, J.L. and Krueger, K.E. (1995) Expression of cytochrome P450 side-chain cleavage enzyme and 3 $\beta$ -hydroxysteroid dehydrogenase in the rat central nervous system: a study by polymerase chain reaction and *in situ* hybridization. *J. Neurochem.* **65**, 528–536.
- Sapolsky, R.M. (1985) Glucocorticoid toxicity in the hippocampus: temporal aspects of neuronal vulnerability. *Brain Res.* **359**, 300–305.
- Sapolsky, R.M., Krey, L.C. and McEwen, B.S. (1986) The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. *Endocr. Rev.* **7**, 284–301.
- Sapolsky, R.M., Stein-Behrens, B.A. and Armanini, M.P. (1991) Long-term adrenalectomy causes loss of dentate gyrus and pyramidal neurons in the adult hippocampus. *Exp. Neural.* **114**, 246–249.

- Sapolsky, R.M., Vogelman, J.H., Orentreich, N. and Altmann, J. (1993) Senescent decline in serum dehydroepiandrosterone sulfate concentrations in a population of wild baboons. *J. Gerontol.* **48**, B196–200.
- Sapsee, A.T. (1997) Cortisol, high cortisol diseases and anti-cortisol therapy. *Psychoneuroendocrinology* **22**, S3–S10.
- Sata, M. and Walsh, K. (1998a) Oxidized LDL activates fas-mediated endothelial cell apoptosis. *J. Clin. Invest.* **102**, 1682–1689.
- Sata, M. and Walsh, K. (1998b) Endothelial cell apoptosis induced by oxidized LDL is associated with the down-regulation of the cellular caspase inhibitor FLIP. *J. Biol. Chem.* **273**, 33103–33106.
- Saunders, A.M., Strittmatter, W.J., Schmechel, D., George-Hyslop, P.H., Pericak-Vance, M.A., Joo, S.H., Rosi, B.L., Gusella, J.F., Crapper-MacLachlan, D.R. and Alberts, M.J. (1993) Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* **43**, 1467–1472.
- Scallen, T.J., Noland, B.J., Gavey, K.L., Bass, N.M., Ockner, R.K., Chanderbhan, R. and Vahouny, G.V. (1985) Sterol carrier protein 2 and fatty acid-binding protein. Separate and distinct physiological functions. *J. Biol. Chem.* **260**, 4733–4739.
- Schmidt, B.M., Gerdes, D., Feuring, M., Falkenstein, E., Christ, M. and Wehling, M. (2000) Rapid, nongenomic steroid actions: A new age? *Front. Neuroendocrinol.* **21**, 57–94.
- Schmitt, J., Pohl, J. and Stunnenberg, H.G. (1993) Cloning and expression of a mouse cDNA encoding p59, an immunophilin that associates with the glucocorticoid receptor. *Gene* **132**, 267–271.
- Schwarz, M., Lund, E.G., Lathe, R., Björkhem, I. and Russell, D.W. (1997) Identification and characterization of a mouse oxysterol 7-hydroxylase cDNA. *J. Biol. Chem.* **272**, 23995–24001.
- Schwarz, S. and Pohl, P. (1992) Steroid hormones and steroid hormone binding globulins in cerebrospinal fluid studied in individuals with intact and with disturbed blood-cerebrospinal fluid barrier. *Neuroendocrinology* **55**, 174–182.
- Schwartz-Bloom, R.D., Miller, K.A., Evenson, D.A., Grain, B.J. and Nadler, J.V. (2000) Benzodiazepines protect hippocampal neurons from degeneration after transient cerebral ischemia: an ultrastructural study. *Neuroscience* **98**, 471–484.
- Seckl, J.R. (1997) 11 $\beta$ -Hydroxysteroid dehydrogenase in the brain: a novel regulator of glucocorticoid action? *Front. Neuroendocrinol.* **18**, 49–99.
- Senda, T., Mita, S., Kaneda, K., Kikuchi, M. and Akaike, A. (1998) Effect of SA4503, a novel sigma1 receptor agonist, against glutamate neurotoxicity in cultured rat retinal neurons. *Eur. J. Pharmacol.* **342**, 105–111.
- Setchell, K.D., Schwarz, M., O'Connell, N.C., Lund, E.G., Davis, D.L., Lathe, R., Thompson, H.R., Weslie Tyson, R., Sokol, R.J. and Russell, D.W. (1998) Identification of a new inborn error in bile acid synthesis: mutation of the oxysterol 7 $\alpha$ -hydroxylase gene causes severe neonatal liver disease. *J. Clin. Invest.* **102**, 1690–1703.
- Sérourgne, C., Lefevre, C. and Chevallier, F. (1976) Cholesterol transfer between brain and plasma in the rat: A model for the turnover of cerebral cholesterol. *Exp. Neurol.* **51**, 229–240.
- Sharkey, J. and Butcher, S.P. (1994) Immunophilins mediate the neuroprotective effects of FK506 in focal cerebral ischaemia. *Nature* **371**, 336–339.
- Shealy, C.N. (1995) A review of dehydroepiandrosterone (DHEA). *Integr Physiol. Behav. Sci.* **30**, 308–313.
- Shi, J., Schulze, S. and Lardy, H.A. (2000) The effect of 7-oxo-DHEA acetate on memory in young and old C57BL/6 mice. *Steroids* **65**, 124–129.

- Silve, S., Dupuy, P.H., Labit-Lebouteiller, C., Kaghad, M., Chalon, P., Rahier, A., Taton, M., Lupker, J., Shire, D. and Loison, G. (1996a) Emopamil-binding protein, a mammalian protein that binds a series of structurally diverse neuroprotective agents, exhibits  $\Delta^8$ - $\Delta^7$ -sterol isomerase activity in yeast. *J. Biol. Chem.* **271**, 22434–22440.
- Silve, S., Leplatot, P., Josse, A., Dupuy, P.H., Lanau, C., Kaghad, M., Dhers, C., Picard, C., Rahier, A., Taton, M., Le Fur, G., Caput, D., Ferrara, P. and Loison, G. (1996b) The immunosuppressant SR 31747 blocks cell proliferation by inhibiting a steroid isomerase in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **16**, 2719–2727.
- Silverstein, A.M., Galigniana, M.D., Kanelakis, K.C., Radanyi, C., Renoir, J.M. and Pratt, W.B. (1999) Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein. *J. Biol. Chem.* **274**, 36980–36986.
- Simard, J., Sanchez, R., Durocher, F., Rheaume, E., Turgeon, C., Labrie, Y., Luu-The, V., Mebarki, F., Morel, Y. and de Launoit, Y. (1995) Structure-function relationships and molecular genetics of the  $3\beta$ -hydroxy steroid dehydrogenase gene family. *J. Steroid. Biochem. Molec. Biol.* **55**, 489–505.
- Simpson, E.R., Zhao, Y., Agarwal, V.R., Michael, M.D., Bulun, S.E., Hinshelwood, M.M., Graham-Lorence, S., Sun, T., Fisher, C.R., Qin, K. and Mendelson, C.R. (1997) Aromatase expression in health and disease. *Recent Prog. Horm. Res.* **52**, 185–213.
- Singh, V.B., Kalimi, M., Phan, T.H. and Boodle-Biber, M.C. (1994) Intracranial dehydroepiandrosterone blocks the activation of tryptophan hydroxylase in response to acute sound stress. *Mol. Cell Neurosci.* **5**, 176–181.
- Slominski, A., Ermak, G. and Mihm, M. (1996) ACTH receptor, CYP11A1, CYP17 and CYP21A2 genes are expressed in skin. *J. Clin. Endocrinol. Metab.* **81**, 2746–2749.
- Sloviter, R.S., Valiquette, G., Abrams, G.M., Ronk, E.G., Sollas, A.L., Paul, L.A. and Neubort, S. (1989) Selective loss of hippocampal granule cells in the mature rat brain after adrenalectomy. *Science* **243**, 535–538.
- Smith, D.F., Baggenstoss, B.A., Marion, T.N. and Rimerman, R.A. (1993) Two FKBP-related proteins are associated with progesterone receptor complexes. *J. Biol. Chem.* **268**, 18365–18371.
- Song, W., Chen, J., Dean, W.L., Redinger, R.N. and Prough, R.A. (1998) Purification and characterization of hamster liver microsomal  $7\alpha$ -hydroxycholesterol dehydrogenase. Similarity to type I  $11\beta$ -hydroxysteroid dehydrogenase. *J. Biol. Chem.* **273**, 16223–16228.
- Song, W.C., Funk, C.D. and Brash, A.R. (1993) Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8519–8523.
- Spohn, M. and Davison, A.N. (1972) Cholesterol metabolism in myelin and other subcellular fractions of rat brain. *J. Lipid Res.* **13**, 563–570.
- Sprengel, R., Werner, P., Seeburg, P.H., Mukhin, A.G., Santi, M.R., Grayson, D.R., Guidotti, A. and Krueger, K.E. (1989) Molecular cloning and expression of cDNA encoding a peripheral-type benzodiazepine receptor. *J. Biol. Chem.* **264**, 20415–20421.
- Stapleton, G., Steel, M., Richardson, M., Mason, J.O., Rose, K.A., Morris, R.G. and Lathe, R. (1995) A novel cytochrome P450 expressed primarily in brain. *J. Biol. Chem.* **270**, 29739–29745.
- Stoka, A.M. (1999) Phylogeny and evolution of chemical communication: an endocrine approach. *J. Mol. Endocrinol.* **22**, 207–225.
- Strigini, M. and Cohen, S.M. (1999) Formation of morphogen gradients in the *Drosophila* wing. *Semin. Cell. Dev. Biol.* **10**, 335–344.
- Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S. and Roses, A.D. (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased

- frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1977–1981.
- Stromstedt, M., Warner, M., Banner, C.D., MacDonald, P.C. and Gustafsson, J.A. (1993) Role of brain cytochrome P450 in regulation of the level of anesthetic steroids in the brain. *Mol. Pharmacol.* **44**, 1077–1083.
- Stromstedt, M. and Waterman, M.R. (1995) Messenger RNAs encoding steroidogenic enzymes are expressed in rodent brain. *Brain Res. Mol. Brain Res.* **34**, 75–88.
- Stuerenburg, H.J., Fries, U., Iglauer, F. and Kunze, K. (1997) Effect of age on synthesis of the GABAergic steroids 5 $\alpha$ -pregnane-3, 20-dione and 5 $\alpha$ -pregnane-3 $\alpha$ -ol-20-one in rat cortex in vitro. *J. Neural. Transm.* **104**, 249–257.
- Stumpf, W.E. (1988) Vitamin D—solatriol the heliogenic steroid hormone: somatotrophic activator and modulator. Discoveries from histochemical studies lead to new concepts. *Histochemistry* **89**, 209–219.
- Su, J., Chai, X., Kahn, B. and Napoli, J.L. (1998) cDNA cloning, tissue distribution, and substrate characteristics of a *cis*-Retinol/3 $\alpha$ -hydroxysterol short-chain dehydrogenase isozyme. *J. Biol. Chem.* **273**, 17910–17916.
- Su, T.P., London, E.D. and Jaffe, J.H. (1988) Steroid binding at sigma receptors suggests a link between endocrine, nervous, and immune systems. *Science* **240**, 219–221.
- Su, T.P., Shukla, K. and Gund, T. (1990) Steroid binding at sigma receptors: CNS and immunological implications. *Ciba Found. Symp.* **153**, 107–116.
- Subramaniam, N., Cairns, W. and Okret, S. (1998) Glucocorticoids repress transcription from a negative glucocorticoid response element recognized by two homeodomain-containing proteins, Pbx and Oct-1. *J. Biol. Chem.* **273**, 23567–23574.
- Subramaniam, N., Treuter, E. and Okret, S. (1999) Receptor interacting protein RIP140 inhibits both positive and negative gene regulation by glucocorticoids. *J. Biol. Chem.* **274**, 18121–18127.
- Sudjic, M.M. and Booth, R. (1976) Activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in brains of adult and 7-day-old rats. *Biochem. J.* **154**, 559–560.
- Sugawara, T., Lin, D., Holt, J.A., Martin, K.O., Javitt, N.B., Miller, W.L. and Strauss, III, J.F., (1995) Structure of the human steroidogenic acute regulatory protein (StAR) gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase activity. *Biochemistry* **34**, 12506–12512.
- Sweet, F., Ahmed, R., Morgan, T.E. and Sweet, B.C. (1980) Bifunctional enzyme activity at the same active site: competitive inhibition kinetics with 3 $\alpha$ /20 $\beta$ -hydroxysteroid dehydrogenase. *Steroids* **35**, 111–118.
- Tchoudakova, A. and Callard, G.V. (1998) Identification of multiple CYP19 genes encoding different cytochrome P450 aromatase isozymes in brain and ovary. *Endocrinology* **139**, 2179–2189.
- Teuber, L., Watjens, F. and Jensen, L.H. (1999) Ligands for the benzodiazepine binding site—a survey. *Curr. Pharm. Des.* **5**, 317–343.
- Thomas, J.L., Evans, B.W., Blanco, G., Mason, J.I. and Strickler, R.C. (1999) Creation of a fully active, cytosolic form of human type I 3 $\beta$ -hydroxy steroid dehydrogenase/isomerase by the deletion of a membrane-spanning domain. *J. Mol. Endocrinol.* **23**, 231–239.
- Thompson, E.B., Medh, R.D., Zhou, F., Ayala-Torres, S., Ansari, N., Zhang, W. and Johnson, B.H. (1999) Glucocorticoids, oxysterols, and cAMP with glucocorticoids each cause apoptosis of CEM cells and suppress c-myc. *J. Steroid Biochem. Molec. Biol.* **69**, 453–461.
- Tint, G.S., Pentchev, P., Xu, G., Batta, A.K., Shefer, S., Salen, G. and Honda, A. (1998) Cholesterol and oxygenated cholesterol concentrations are markedly elevated in peripheral tissue but not in brain from mice with the Niemann-Pick type C phenotype. *J. Inherit. Metab. Dis.* **21**, 853–863.

- Todaro, G.J., Rose, T.M. and Shoyab, M. (1991) Human DBI (endozepine): relationship to a homologous membrane associated protein (MA-DBI). *Neuropharmacology* **30**, 1373–1380.
- Tong, Y., Toranzo, D. and Pelletier, G. (1991) Localization of diazepam-binding inhibitor (DBI) mRNA in the rat brain by high resolution *in situ* hybridization. *Neuropeptides* **20**, 33–40.
- Toran-Allerand, C.D., Singh, M. and Setalo, Jr. G. (1999) Novel mechanisms of estrogen action in the brain: new players in an old story. *Front. Neuroendocrinol.* **20**, 97–121.
- Uittenbogaard, A., Ying, Y. and Smart, E.J. (1998) Characterization of a cytosolic heat-shock protein-caveolin chaperone complex. Involvement in cholesterol trafficking. *J. Biol. Chem.* **273**, 6525–6532.
- Ukena, K., Kohchi, C. and Tsutsui, K. (1999) Expression and activity of 3 $\beta$ -hydroxy steroid dehydrogenase/delta5-delta4-isomerase in the rat Purkinje neuron during neonatal life. *Endocrinology* **140**, 805–813.
- Urani, A., Privat, A. and Maurice, T. (1998) The modulation by neurosteroids of the scopolamine-induced learning impairment in mice involves an interaction with sigma1 (sigma1) receptors. *Brain Res.* **799**, 64–77.
- Uzunova, V., Sheline, Y., Davis, J.M., Rasmusson, A., Uzunov, D.P., Costa, E. and Guidotti A. (1998) Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 3239–3244.
- van der Wielen, R.P., Lowik, M.R., van den Berg, H., de Groot, L.C., Haller, J., Moreiras, O. and van Staveren, W.A. (1995) Serum vitamin D concentrations among elderly people in Europe. *Lancet* **346**, 207–210.
- Van Uden, E., Veinbergs, L., Mallory, M., Orlando, R. and Masliah, E. (1999) A novel role for receptor-associated protein in somatostatin modulation: implications for Alzheimer's disease. *Neuroscience* **88**, 687–700.
- Vanier, M.T., Suzuki, K., Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M.A., *et al.* (1997) Recent advances in elucidating Niemann-Pick C disease Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* **277**, 228–231.
- Vatassery, G.T., Quach, H.T., Smith, W.E., Krick, T.P. and Ungar, F. (1997) Analysis of hydroxy and keto cholesterol in oxidized brain synaptosomes. *Lipids* **32**, 101–107.
- Vilner, B.J., de Costa, B.R. and Bowen, W.D. (1995) Cytotoxic effects of sigma ligands: Sigma receptor-mediated alterations in cellular morphology and viability. *J. Neurosci.* **15**, 117–134.
- Vreugdenhil, E., de Jong, J., Schaaf, M.J., Meijer, O.C., Busscher, J., Vuijst, C. and De Kloet, E.R. (1996) Molecular dissection of corticosteroid action in the rat hippocampus. Application of the differential display techniques. *J. Mol. Neurosci.* **7**, 135–146.
- Wagner, B.L., Pollio, G., Giangrande, P., Webster, J.C., Breslin, M., Mais, D.E., Cook, C.E., Vedeckis, W.V., Cidlowski, J.A. and McDonnell, D.P. (1999) The novel progesterone receptor antagonists RTI 3021–012 and RTI 3021–022 exhibit complex glucocorticoid receptor antagonist activities: implications for the development of dissociated antiprogestins. *Endocrinology* **140**, 1449–1458.
- Wan, C.P. and Lau, B.H. (1995) Neuropeptide Y receptor subtypes. *Life Sci.* **56**, 1055–1064.
- Warner, M., Stromstedt, M., Moller, L. and Gustafsson, J.A. (1989) Distribution and regulation of 5 alpha-androstane-3 $\beta$ , 17 $\beta$ -diol hydroxylase in the rat central nervous system. *Endocrinology* **124**, 2699–2706.
- Wassif, C.A., Maslen, C., Kachilele-Linjewile, S., Lin, D., Linck, L.M., Connor, W.E., Steiner, R.D. and Porter, F.D. (1998) Mutations in the human sterol  $\Delta^7$ -reductase gene at 11q12–13 cause Smith-Lemli-Opitz syndrome. *Am. J. Hum. Genet.* **63**, 55–62.



- Watari, H., Arakane, F., Moog-Lutz, C., Kallen, C.B., Tomasetto, C., Gerton, G.L., Rio, M.C., Baker, M.E. and Strauss, J.F., 3rd. (1997). MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8462–8467.
- Waterham, H.R., Wijburg, F.A., Hennekam, R.C., Vreken, P., Poll-The, B.T., Dorland, L., Duran, M., Jira, P.E., Smeitink, J.A., Wevers, R.A. and Wanders, R.J. (1998) Smith-Lemli-Opitz syndrome is caused by mutations in the 7-dehydrocholesterol reductase gene. *Am. J. Hum. Genet.* **63**, 329–338.
- Watters, D. and Lavin, M. (1999) *Signalling Pathways in Apoptosis*. Harwood Academic, Amsterdam.
- Waxman, D.J. (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch. Biochem. Biophys.* **369**, 11–23.
- Webb, N.R., Rose, T.M., Malik, N., Marquardt, H., Shoyab, M., Todaro, G.J. and Lee, D.C. (1987) Bovine and human cDNA sequences encoding a putative benzodiazepine receptor ligand. *DNA* **6**, 71–79.
- Wehling, M., Christ, M. and Gerzer, R. (1993) Aldosterone-specific membrane receptors and related rapid, non-genomic effects. *Trends Pharmacol. Sci.* **14**, 1–4.
- Weier, R.M. and Hofmann, L.M. (1975) 7 $\alpha$ -Carboalkoxy steroidal spirolactones as aldosterone antagonists. *J. Med. Chem.* **18**, 817–821.
- Wersinger, S.R., Sannen, K., Villalba, C., Lubahn, D.B., Rissman, E.F. and De Vries, G.J. (1997) Masculine sexual behavior is disrupted in male and female mice lacking a functional estrogen receptor  $\alpha$  gene. *Horm. Behav.* **32**, 176–183.
- Whalin, M.E., Boujrad, N., Papadopoulos, V. and Krueger, K.E. (1994) Studies on the phosphorylation of the 18kDa mitochondrial benzodiazepine receptor protein. *J. Recept. Res.* **14**, 217–228.
- Whiting, P.J., Bonnert, T.P., McKernan, R.M., Farrar, S., Le Bourdelles, B., Heavens, R.P., Smith, D.W., Hewson, L., Rigby, M.R., Sirinathsinghji, D.J., Thompson, S.A. and Wafford, K.A. (1999) Molecular and functional diversity of the expanding GABA-A receptor gene family. *Ann. NY Acad. Sci.* **868**, 645–653.
- Wietholtz, H., Marschall, H.U., Sjovall, J. and Matern, S. (1996) Stimulation of bile acid 6 $\alpha$ -hydroxylation by rifampin. *J. Hepatol.* **24**, 713–718.
- Willnow, T.E., Hilpert, J., Armstrong, S.A., Rohlmann, A., Hammer, R.E., Burns, D.K. and Herz, J. (1996) Defective forebrain development in mice lacking gp330/megalin. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8460–8464.
- Wolf, O.T., Koster, B., Kirschbaum, C., Pietrowsky, R., Kern, W., Hellhammer, D.H., Born, J. and Fehm, H.L. (1997a) A single administration of dehydroepiandrosterone does not enhance memory performance in young healthy adults, but immediately reduces cortisol levels. *Biol. Psychiatry* **42**, 845–848.
- Wolf, O.T., Kudielka, B.M., Hellhammer, D.H., Hellhammer, J. and Kirschbaum, C. (1998a) Opposing effects of DHEA replacement in elderly subjects on declarative memory and attention after exposure to a laboratory stressor. *Psychoneuroendocrinology* **23**, 617–629.
- Wolf, O.T., Naumann, E., Hellhammer, D.H. and Kirschbaum, C. (1998b) Effects of dehydroepiandrosterone replacement in elderly men on event-related potentials, memory, and well-being. *J. Gerontol. A Biol. Sci. Med. Sci.* **53**, M385–M390.
- Wolf, O.T., Neumann, O., Hellhammer, D.H., Geiben, A.C., Strasburger, C.J., Dressendorfer, R.A., Pirke, K.M. and Kirschbaum, C. (1997b) Effects of a two-week physiological dehydroepiandrosterone substitution on cognitive performance and well-being in healthy elderly women and men. *J. Clin. Endocrinol. Metab.* **82**, 2363–2367.

- Woods, M.J. and Williams, D.C. (1996) Multiple forms and locations for the peripheral-type benzodiazepine receptor. *Biochem. Pharmacol.* **52**, 1805–1814.
- Wu, F.S., Gibbs, T.T. and Farb, D.H. (1991) Pregnenolone sulfate: a positive allosteric modulator at the N-methyl-D-aspartate receptor. *Mol. Pharmacol.* **40**, 333–336.
- Xu, G., Servatius, R.J., Shefer, S., Tint, G.S., O'Brien, W.T., Batta, A.K. and Salen, G. (1998) Relationship between abnormal cholesterol synthesis and retarded learning in rats. *Metabolism* **47**, 878–882.
- Yamada, J., Sugiyama, H., Sakuma, M. and Suga, T. (1994) Specific binding of dehydroepiandrosterone sulfate to rat liver cytosol: a possible association with peroxisomal enzyme induction. *Biochim. Biophys. Acta* **1224**, 139–146.
- Yardin, C., Terro, F., Lesort, M., Esclaire, F. and Hugon, J. (1998) FK506 antagonizes apoptosis and c-jun protein expression in neuronal cultures. *Neuroreport* **9**, 2077–2080.
- Yoo, A., Harris, J. and Dubrovsky, B. (1996) Dose-response study of dehydroepiandrosterone sulfate on dentate gyrus long-term potentiation. *Exp. Neurol.* **137**, 151–156.
- Young, J., Corpechot, C., Perche, F., Eychenne, B., Haug, M., Baulieu, E.E. and Robel, P. (1996) Neurosteroids in the mouse brain: behavioral and pharmacological effects of a 3 $\beta$ -hydroxy steroid dehydrogenase inhibitor. *Steroids* **61**, 144–149.
- Yu, Y., Li, W., Su, K., Yussa, M., Han, W., Perrimon, N. and Pick, L. (1997) The nuclear hormone receptor Ftz-F1 is a cofactor for the Drosophila homeodomain protein Ftz. *Nature* **385**, 552–555.
- Zhang, H., LeCulysse, E., Liu, L., Hu, M., Matoney, L., Zhu, W. and Yan, B. (1999) Rat pregnane X receptor: molecular cloning, tissue distribution, and xenobiotic regulation. *Arch. Biochem. Biophys.* **368**, 14–22.
- Zhang, J., Akwa, Y., el-Etr, M., Baulieu, E.E. and Sjoval, J. (1997a) Metabolism of 27-, 25- and 24-hydroxycholesterol in rat glial cells and neurons. *Biochem. J.* **322**, 175–184.
- Zhang, J., Xue, Y., Jondal, M. and Sjoval, J. (1997b) 7 $\alpha$ -Hydroxylation and 3-dehydrogenation abolish the ability of 25-hydroxycholesterol and 27-hydroxycholesterol to induce apoptosis in thymocytes. *Eur. J. Biochem.* **247**, 129–135.
- Zhao, H.F., Labrie, C., Simard, J., de Launoit, Y., Trudel, C., Martel, C., Rheume, E., Dupont, E., Luu-The, V. and Pelletier, G. (1991) Characterization of rat 3 $\beta$ -hydroxy steroid dehydrogenase/ $\Delta^5$ - $\Delta^4$  isomerase cDNAs and differential tissue-specific expression of the corresponding mRNAs in steroidogenic and peripheral tissues. *J. Biol. Chem.* **266**, 583–593.
- Zwain, I.H. and Yen, S.S. (1999a) Dehydroepiandrosterone: biosynthesis and metabolism in the brain. *Endocrinology* **140**, 880–887.
- Zwain, I.H. and Yen, S.S. (1999b) Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. *Endocrinology* **140**, 3843–3852.

# INDEX

- Accessory proteins 6–8
- Acyl CoA
  - cholesterol acyl transferase (ACAT) 123
  - hydratase/dehydrogenase 85, 86
  - oxidase 85, 86
  - thiolase 85, 86
- Adrenarche 272–4
- Adrenodoxin 3, 6, 23, 148, 181, 414
- Adrenodoxin reductase 3, 6, 23, 181
- Adrenopause 272–4
- Albumin D-site binding protein (DBP) 93–4
- Alcohol dehydrogenase 85, 86
- Aldehyde dehydrogenase 85, 86
- Aldoketoreductases (AKR) 5
- Aldosterone synthase (*see* CYP11B2)
- Androgens 2
  - sex differentiation 299–300
- Angiotensin II receptor 184–5
- Apparent mineralocorticoid excess (AME) 345–6
- Aromatase (*see* CYP 19)
- adrenal-specific protein (ASP) 152
- Atherosclerosis 102
- Basic helix-loop-helix-leucine zipper (bHLH-Zip) proteins 46
- Bile acid
  - binding protein 82
  - biosynthesis 81–104
    - genetic defects 99–104
  - CoA ligase 85, 86
  - pathways 83
  - response element (BARE) 93
  - response protein (BARP) 95
  - transporter 82–3
- C4A 148–51, 153, 157–9
- C4B 148–50
- CAR- $\beta$  425, 439
- Caveolae 119
- Caenorhabditis elegans 37
- Cerebrotendinous xanthomatosis 61, 99, 102, 433, 447
- Cholesterol
  - cell death 436–7
  - homeostasis 81–3
  - 7 $\alpha$ -hydroxylase (*see* CYP7A)
  - metabolism (steroid) 115–37
  - side-chain cleavage enzyme (*see* CYP11A)
  - synthesis 10, 37–51, 119–23
  - transport 8–10, 21, 121–3
  - intramitochondrial 127–36
- Cholesterol ester
  - formation 123
  - hydrolase (neutral) 125
  - hydrolysis
    - extralysosomal 125
    - lysosomal 123–5
  - storage disease 124–5
- Cofactors 6–8
- Cholic acid biosynthesis 83, 85
  - alternative (acidic) 83, 84, 86
  - classical (neutral) 85–6
  - regulation of 86–7

- Chromatin 388–9
- Congenital adrenal hyperplasia 154–67, 190–5, 222, 242
- Congenital lipoid adrenal hyperplasia 128–31
- Coregulator function diversity of mechanisms 391–2
- COUP-TFII 91–5
- CREM 42
- CREB-binding protein (CBP) 385–7
  - coactivators 383–6
  - cointegrators 386–7
  - corepressors 387–8
- Cyclo-oxygenase 2
- Cyclophilins 433, 437
- CYP superfamily 2
- CYP2B4 263
- CYP2B6 61, 83, 87
- CYP4A 147
- CYP7A1 81, 82, 84, 85, 88, 89–99, 100–1
  - activity regulation 91–5
  - deficiency 100–3
  - gene structure 88
  - transcriptional regulation 91–4
- CYP7B1 86, 88, 97–8, 101
  - deficiency 99–101
  - gene structure 88
- CYP8B1 84–5, 88, 98–9
  - gene structure 88
- CYP11A 3, 5–7, 8–10, 21–3, 117–18, 120, 128, 147–8, 412, 414, 419, 428
  - deficiency 22, 362–3
- CYP11B 179–86
  - deficiency 12–14
  - mutation frequencies 200–1
  - transcriptional regulation 186–90
- CYP11B1 3, 4, 10, 16, 17, 180–201
  - deficiency 190–2
  - gene 181–3
  - gene regulation 183–6
- CYP11B2 3–4, 59, 62, 180–201
  - allelic variations 199–200
  - brain 422–3
  - deficiency 192–6
  - gene 181–3
  - gene regulation 183–4
    - angiotensin II 184
    - potassium 185
  - ACTH 185–6
- CYP17 3–4, 7, 9–20, 23, 72, 117, 120, 259–81
  - brain 415
  - deficiency 276–81
    - combined 276–8
    - isolated lyase 278–81
  - mechanism of catalysis 262–3
  - modeling 263–9
  - one enzyme or two? 261–2
  - physiology 271–4
  - redox partners 269–71
- CYP19 4, 6, 7, 16, 18–20, 23–4, 117, 120, 287–90
  - brain 410–11
  - gene structure 263
  - deficiency
    - humans 289–92
    - knockout (ArKO) mouse 292–4
    - lipid/carbohydrate phenotype 292–3
  - testicular differentiation 289–92
- CYP21 3, 4, 7, 16–17, 145–68
  - deficiency 13, 149, 154–67
    - detection 163–5
    - diagnosis 163–5
    - genotype/phenotype 165–7
    - linked microsatellites 165
    - mouse model 363
    - salt-wasting 154–5
    - virilization 155–6
  - gene structure 148–50
  - HLA linkage 156–7
  - mutations 157–62
    - detection 163–5
  - polymorphisms 162
  - recombinations 163
  - structure-function 146–8
  - transcription 151–3
- CYP21P 148–50, 151, 157, 158, 159
- CYP24 59, 62, 95
- CYP27A1 58–61, 84–86, 88, 95–96, 433
  - deficiency 61, 102–3, 432–3
  - gene structure 88
- CYP27B1 57, 63–4, 67, 95
  - deficiency (VDDR-I) 64–71
  - structure-function correlations 71–4
- CYP39A1 433
- CYP46 433, 442
- CYP51 41–3

- CYP52 147  
 CYP101 59, 146, 263  
 CYP102 263, 264  
 CYP107A1 59  
 CYP 108 59  
 Cytochrome b<sub>5</sub> 17, 60, 269–70, 280  
 Cytochrome P450 2
- DAX-1 371  
 Dehydroepiandrosterone 412–14  
     brain metabolism 441–6  
 7 $\alpha$ -Dehydrogenase 86  
 5'-Deiodinase 339  
 Desmosterolosis 51  
*Drosophila melongaster* 38
- Emopamil binding protein 429, 430–1  
 Estrogen 2  
     receptor 371–2, 381–4, 425, 426
- Familial hypercholesterolemia 122  
 Ferredoxin (*see* Adrenodoxin)  
 Fushi tarazu (Ftz) gene 447–8
- GABA receptor 426–7  
 Glucocorticoid 2, 179–201, 339–43, 426  
     action/modulation in brain 443–4  
     receptor 371–2  
 Guanylate cyclase 2
- Holoprosencephaly 448  
 HDL receptors 122  
     SR-B1 122, 123  
     mouse 'knockout' 359  
     CLA-1 123  
 HE1 gene 126  
 Hke6 gene 310  
 HMGCoA biosynthesis 39  
 HMGCoA reductase 40–1, 81, 87, 90, 117,  
     120, 122, 124  
 HNF1 91, 92, 93  
 HNF3 91, 92, 93  
 HNF4 92, 93, 95  
 1 $\alpha$ -Hydroxylase (*see* CYP27B1)  
 7 $\alpha$ -Hydroxylase (*see* CYP7A & 7B)  
 11 $\beta$ -Hydroxylase (*see* CYP11B1)  
 17-Hydroxylase (*see* CYP17)
- 17-Hydroxylase/17, 20-lyase (*see* CYP 17)  
 21-Hydroxylase (*see* CYP21)  
 24-Hydroxylase (*see* CYP24)  
 25-Hydroxylase (*see* CYP27A1)  
     microsomal (*see* CYP2B6)  
 Hydroxysteroid dehydrogenases 4  
 3 $\alpha$ -Hydroxysteroid dehydrogenase 5, 85, 315  
     brain 415–18  
 3 $\beta$ -Hydroxysteroid dehydrogenase/ isomerase  
     5, 101, 209–46  
     biochemistry 210–11  
     brain 415–18  
     human genes/pseudogenes 211–13  
     ontogeny 217–22  
     phylogeny 213–17  
      $\Delta^5$ -C<sub>27</sub>-sterol 85–7, 99, 101  
     deficiency 216  
     type I 5  
     type II 5  
     deficiency (*see* below)  
 3 $\beta$ -Hydroxysteroid dehydrogenase/isomerase  
     (type II) deficiency 222–46  
     biological diagnosis 233–5  
     classical 233–4  
     non-classical 234–5  
     clinical features 223–33  
     non-salt-wasting 223  
     salt-wasting 223  
     genotype/phenotype relations 242–4  
     molecular diagnosis 235–7  
     mutant proteins 237–42  
     sequence variants in HSD3B2 gene 245–6  
     structure-function relations 244–5  
 11 $\beta$ -Hydroxysteroid dehydrogenase 5, 339–51,  
     423–4  
     enzymology 340–3  
     genes 343–52  
     type 1 340–2, 423–4  
     human deficiency 344  
     mouse 'knockout' 345  
     type 2 5, 342–3, 423–4  
     candidate hypertension gene 350–1  
     deficiency 343–51  
     AME 'type II' 349–50  
     apparent mineralocorticoid excess  
     (AME) 345–51  
     mineralocorticoid receptor 347  
     mouse 'knockout' 345

- mutant evolution 349
- 17 $\beta$ -Hydroxysteroid dehydrogenase 5, 300–14, 351–2
  - deficiency 297–314
  - gene family 300–7
  - type 1 5, 300–8
  - type 2 308
  - type 3 298, 300–8
    - deficiency 223, 298–308
    - biological diagnosis 311–12
    - clinical features 310–11
    - management 314
    - molecular diagnosis 312–14
  - type 4 308
  - type 5 308, 311
  - type 6 308
  - type 7 308
  - type 8 310
- 20-Hydroxysteroid dehydrogenase 5
- Hyperaldosteronism 196–201
  - glucocorticoid-suppressible 196–9
- Hyperphosphatemia 67
- Intracellular targets for apoptosis induction 438–9
- Jun/API 312
- Keratinocytes 63–4, 66
- Kidney 57, 59
- 'Knockouts' 21–3, 292–4, 359–66
- Lamin B receptor 43
- Lanosterol 14 $\alpha$ -demethylase (*see* CYP51)
- Lysosomal acid lipase 124–5
- LDL 121
  - receptor 81, 87, 118, 121
  - related protein (LRP) 81, 432
- LXR (liver orphan receptor) 91, 439
- MAS sterols 40, 42, 43, 44
- Membrane associated steroid receptors 425–6
- Mineralocorticoid 2, 179–201, 339, 345–7
- Mycobacterium smegmatis 37
- Neurosteroids 408–51
  - action at neurotransmitter receptors 426–7
  - aging link 443–6
- Niemann-Pick type C disease 125–7, 407, 432, 447
  - NPC1 gene 126
- NCoR 385, 387–8
- NF- $\kappa$ B 384, 387, 393
- NGFI B 153
- NMDA type channel receptor 426
- Nuclear receptor family
  - brain 423–4
  - diversity, evolutionary aspects 373
  - mechanism of action 375–88
    - alternate mechanisms 392–4
    - chaperone and synthesis 375–6
    - DNA binding 376–81
    - ligand binding 381–3
  - nomenclature 373–4
  - regulation of expression 369–94
  - structural features 371–2
- OPPI-6 412
- Oxidoreductases 2
- Oxysterol 87
  - binding protein (OSBP) 414
  - 7 $\alpha$ -hydroxylase (*see* CYP7B1)
  - metabolism in brain 433
  - receptor (apoptotic) 437–9
  - signaling 433
- P450aldo (*see* CYP11B2)
- P450arom (*see* CYP19)
- P450BMP (*see* CYP102)
- P450cam (*see* CYP101)
- P450c1 $\alpha$  (*see* CYP27B)
- P450c11 (*see* CYP11B1)
- P450c17 (*see* CYP17)
- P450c21 (*see* CYP21)
- P450c24 (*see* CYP24)
- P450scc (*see* CYP11A)
- P450 reductase 6, 15, 42, 145, 288, 414
- Patched (Ptc) 126, 447
- Parturition 23–25
- Perilipins 118
- Peripheral benzodiazapine receptor (PBR) 407, 428–9
- Peroxisomal disorders 103–4

- Peroxisomal proliferator activated receptor (PPAR) 377
- Phosphorylation 8, 9
- Post-squalene pathway 49–51
- Pregnancy 23–5
- Prostaglandins 42
- Pseudohermaphroditism, male 317
- 5 $\alpha$ -Reductase  
   gene family 315–17  
   structure-function 315–16  
   type 1 315  
   type 2 315  
     polymorphisms/prostate cancer 317  
   type 2 deficiency 223, 299, 314, 317, 320–1, 327, 329  
     biological diagnosis 320  
     clinical features 317–20  
     management 329–30  
     molecular diagnosis 320–9
- 5 $\beta$ -Reductase 85  
   deficiency 99, 100, 101–2
- Retinoic acid receptor (RAR) 377, 387, 389
- Retinoid X receptor (RXR) 377
- Retinol dehydrogenase 308
- Rifampicin 446
- SF-1 (steroidogenic factor 1) 134, 152, 186, 188, 364, 381, 414  
   Mouse 'knockout' 359–60
- Sigma receptor 44–5, 429–31  
   ligand 429–30
- Short-chain dehydrogenase/reductase (SDR) 5
- Site 1 protease (SIP) 47
- Site 2 protease (S2P) 47
- Smith-Lemli-Opitz syndrome (SLOS) 39, 45, 49–51, 100, 103, 115, 120, 412, 432, 447, 450
- SMRT 385–7
- Sonic hedgehog (Sh) gene 37, 39, 50, 447
- Spl 92
- Squalene pathway 39–41
- Squalene synthetase 124
- SREBP 37, 39, 42  
   regulation 42, 46–9  
   activating cleavage protein (SCAP) 47, 126
- Steroids  
   Apoptosis and neurodegeneration 436–43  
   brain development and function 407  
   cell death 436  
   masculinization of brain function 407
- Steroid biosynthesis  
   adrenal 179–81  
   brain 412–19  
   secondary defects 365
- Steroid metabolism  
   brain function 431–3  
   in peripheral tissues 339–52
- Steroid receptor coactivators 384  
   P160 family 384–6, 389
- Steroidogenic acute regulatory protein (StAR) 8, 128–36, 414  
   gene 128–30  
   homologues 134  
   human deficiency 21, 128–31  
   mouse 'knockout' 359–60  
   post-translational modification 133  
   structure-function 146–8  
   transcriptional regulation 134–5
- Steroidogenic cell structure 115–22
- Sterol  
   biosynthesis 37–51  
   brain 407–51  
   C5-desaturase 45  
   4,4-demethylase 44  
   12 $\alpha$ -hydroxylase (*see* CYP 8B1)  
   27-hydroxylase (*see* CYP 27A1)  
    $\Delta^{8,7}$ -isomerase 44–45  
    $\Delta^7$ -reductase 45, 49, 50  
     deficiency (*see* Smith-Opitz-Lemli syndrome)  
    $\Delta^{14}$ -reductase 43  
    $\Delta^{24,25}$ -reductase 25, 45, 46, 51
- Thyroid receptor associated proteins (TRAP) 389
- Vitamin D  
   biosynthesis 57–74  
   binding protein 58  
   -dependent rickets type I (VDDR-I) 64, 66–9  
   disorders 57  
   receptor 375

-interacting protein (DRIP) 389

Wolman's disease 124

Zellweger syndrome 103–4

Zonation 16–20, 179